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CANCER OF THE PENIS AND CIRCUMCISION IN RELATION TO THE INCUBATION PERIOD OF CANCER.

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Some items of the literature summarized below are already well known to those engaged in cancer research, but the inferences about pre-cancerous changes to be drawn from these and other data are so important that it seems best to present all the material together. No attempt is made here to review the literature about the incubation period of other forms of cancer in man, nor to discuss the indications of experimental work on this period in animals.

There is a considerable literature upon the relation of cancer of the penis to circumcision among Jews, who circumcise on the eighth day, and among Moslems, who circumcise between the third and fourteenth years.

1. *Circumcision among Jews.*

The Mosaic law enacts (Leviticus, 12, 2–3) that "If a woman conceive seed, and bear a man child, then she shall be unclean seven days; as in the days of the impurity of her sickness shall she be unclean. And in the eighth day the flesh of his foreskin shall be circumcised." Talmudic writers lay down that the operation must not be deferred even though the eighth day is the Sabbath (Preuss, 1923), and may be postponed only if the child is ill.

2. *Circumcision among Moslems.*

In contrast to the Jewish practice, one may quote a description by Alfred Russell Wallace of the operation as he saw it among Moslems in Java (1898). " . . . the two lads, who were about fourteen years old, were brought out clothed in a sarong from the waist downwards, and having the whole body covered with a yellow powder, and profusely decked with white blossoms in wreaths, necklaces and armlets, looking at first sight very like savage brides. They were conducted by two priests to a bench placed in front of the house in the open air, and the ceremony of circumcision was then performed before the assembled crowd."

Lane (1842), writing of Egypt, says: " . . . at the age of about 5 or 6 years or sometimes later, the boy is circumcised," while the operation is carried

out "among the peasants not infrequently at the age of 12, 13 or 14 years." Among Moslems in India the usual age is between the 6th and 9th years (V. R. Khanolkar, private communication). Kouwenaar (1933) states that in Java the operation is carried out usually between the 10th and 14th years; but in West Java earlier, between the 3rd and 6th years.

3. *Circumcision in Relation to Cancer of the Penis.*

(a) *Jewish circumcision.*

Sorsby (1931) examined the records of 2252 deaths from cancer in male Jews (1073 in London, 1910–1925, and 1179 in Vienna, 1921–1927) and did not find a single case of cancer of the penis among them, although 12 cases were to be expected among the same numbers of the general population. Wolbarst (1932) obtained records of cases of this form of cancer for the period 1925–30 from a number of hospitals in the United States, with the following results:

> 830 cases, all in non-Jews, from 179 hospitals having 4·4 per cent Jewish patients.
>
> 7 cases, all in non-Jews, from 26 Jewish hospitals having 73 per cent Jewish patients.
>
> "One case of penile cancer in a Jew was reported by a mid-west hospital, but this man had not been circumcised."* This instance serves to show that Jews have no inherent immunity to this form of cancer.

Wolbarst, by adding his own data to those of five other authors, obtains a total of 1103 cases in the United States without a single one in a circumcised Jew, although Jews make up about 3 per cent of the population.

Föderl (1926) collected the data relating to 276 operations for carcinoma on males at a Jewish hospital ("Spital der israelitischen Kulturgemeinde") in Vienna during 11 years. There was no case of cancer of the penis, although the author reckons that 13 cases (4·7 per cent) would be expected in a similar sample of the general population.

Wolff (1939) found no case of cancer of the penis among 726 deaths of male Jews from cancer in Berlin in 6 years (1924–26, 1932–34). Dean (1935) found no Jew in his series of 120 cases of cancer of the penis at the Memorial Hospital, New York, although a large proportion of the patients at that hospital are Jews. In a later paper Dean (1936) records the case of a Jew, aged 66, in whom the prepuce had been removed completely, presumably on the eighth day, who suffered from obstruction to the outflow of urine, and developed a carcinoma close to the urethral aperture. Sections showed that "the tumour began in multiple foci about the meatus . . . the disease apparently arose from the urinary stream irritating a tight external urethral meatus." This seems to be the only recorded case of carcinoma of the penis in a circumcised Jew, and the very unusual site of the growth was such that the presence or absence of a prepuce could not well have any effect.

* Wolbarst does not give any details of this case. It is possible for an orthodox Jew to be uncircumcised. A Talmudic writer lays down that if two sons (or three, according to another authority) of the same mother, or a son of each of three sisters, have died as a result of circumcision, the next son, or the son of the fourth sister, shall not be circumcised (Preuss, p. 285). Probably fatalities in haemophilic families gave rise to these exemptions.

(b) *Moslem circumcision.*

There seems to be very little information in medical literature about the technique and results of Moslem circumcision. In spite of the immense mass of material presented by the Moslem populations of the world, numbering about 100 million males, we have no data for individuals which would enable us to compare the effects of circumcision earlier, and later, in the range of 3 to 14 years of age. Such data, on a statistical basis, would be of great interest.

Wolbarst (1932) obtained from hospitals in India (Madras Presidency, Madras City, Government of Bengal, Central Provinces, Assam) the following data for 1925–30:

TABLE I—*Cancer of the Penis in Hindus and Moslems.* (Wolbarst.).

Total cancers in men.	Cancer of penis.			Percentage of Moslems in hospitals.
	Hindu.	Moslem.	Total.	
7692	1169	24	1200	21

The same contrast between the Hindu and Moslem populations of India is shown by the data collected by Nath and Grewal (1935) from 14 hospitals in Punjab, Delhi, Bihar and Orissa, and United Provinces (Table II).

TABLE II—*Cancer of the Penis in Hindus and Moslems.* (Nath and Grewal.).

	Number of cases.		
	Cancer of penis.	Total carcinomas (excluding sarcomas).	Ratio.
Hindu	446	1745	1 : 3·9
Moslem	15	515	1 : 34·3

Dr. V. R. Khanolkar (private communication) gives the following data derived from the first 10,000 cases seen at the Tata Memorial Hospital, Bombay:

TABLE III—*Cancer of the Penis. Cases at the Tata Memorial Hospital.* (Khanolkar.).

	Hindus.			
	Deccani.	Gujerati.	Other.	Muslims.
Carcinoma of penis	57	7	22	2
Total carcinomas in males	969	1086	456	835
Total male patients	1680	1496	803	1269

Megaw (1905), in a series of cases in a hospital in Calcutta, where the admissions of Hindus were less than three times those of Moslems, found 64 cases in Hindus and 2 in Moslems. Kretschmer (1918) in a lecture on cancer of the penis says: "Professor Djeniel Pacha operated 5 carcinomas in four years. He is connected with the Military School at Constantinople which admits only Musselmen," but gives no further information. Sutherland (1904) records that in 12 years at the Mayo Hospital, Lahore, Punjab, where the total admissions of Hindus and Moslems are about equal, there were 72 cases of cancer of the penis in the former

and apparently none in the latter, though the statement on the latter point is not perfectly clear. Kouwenaar (1933) estimates that this form of cancer may contribute as much as 1·5 per cent of all cancers in male Moslems in British India. Cancer of the penis is rather frequent among the Javanese, who are Moslems; Kouwenaar estimates the frequency of this form of cancer to be as much as from 4·4 to 12 per cent of all cancers in males. But he points out that there is doubt whether some of the individuals affected may not be Christians and uncircumcised.

The explanation might be put forward that the occurrence of this form of cancer in Moslems, in contrast to Jews, is due to the less complete removal of the prepuce in an operation carried out later in life. Comparative data upon the completeness of Jewish, and Moslem, circumcision would be of great interest. This factor might act in two ways:

(*a*) By the less complete removal of the cancer-bearing area. If this is the explanation, the cancers which occur in Moslems should arise on a residual portion of the prepuce which would have been removed in a Jewish circumcision, and we have no evidence that this is the case. Moreover, there is certainly no indication that the persons in Dean's series (Table IV, and Fig. 1), nor in that of Schrek

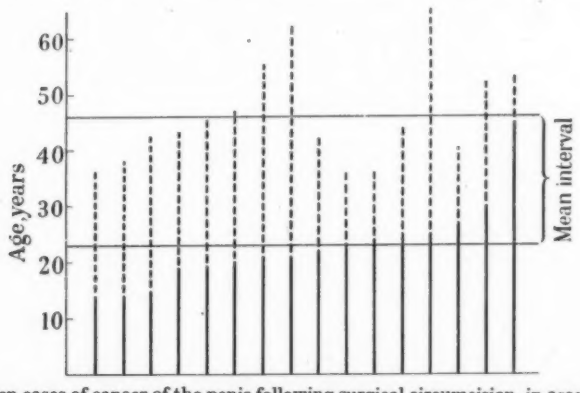

FIG. 1.—Sixteen cases of cancer of the penis following surgical circumcision, in ascending order of age at the time of operation. The interrupted lines represent the interval between the operation and the diagnosis of cancer (Dean; Lewis; Lenowitz and Graham).

and Lenowitz (1947) (Table V), in which cancer of the penis arose, as in Moslems, after circumcision, had been operated upon in any incomplete manner.

(*b*) By allowing the retention of carcinogenic material behind the residual prepuce.

(c) *Surgical circumcision.*

The conclusions to be drawn from the mass statistics of the Mohammedan and Jewish peoples are confirmed by the exact observations upon individuals by Dean (1935, Table IV and Fig. 1) in a study of 120 cases of epithelioma of the penis treated at the Memorial Hospital, New York. He records 7 cases in which cancer of the penis developed on an average 22 years after circumcision performed

at ages from 14 to 45. "In the years following circumcision none of these men had cause for thinking that anything was wrong until a tumour began to grow."

Ngai (1933) records a case of cancer of the penis in a Chinaman, aged 53, who had been circumcised 15 years earlier, but gives no details. Lewis (1931) mentions a case, at Johns Hopkins Hospital, of "a Gentile circumcised at 21 years of age who developed a squamous cell epithelioma at 62 years, beginning as a non-inflammatory sore arising on the corona glandis near the frenum" (Fig. 1). Anderson (1918) met with a case in a man, aged 45, who had been circumcised "when a boy," but he does not state the age at which the operation was performed.

TABLE IV.—*Interval between Circumcision and appearance of Cancer of the Penis.* (Dean, Lewis, Lenowitz and Graham.)

Author.		Age at time of circumcision.	Age when cancer appeared.	Interval (years).
Dean		14	38	24
,,		19	43	24
,,		20	47	27
Lewis		21	62	41
Dean		23	36	13
,,		25	65	40
,,		25	44	19
,,		45	53	8
Average		24	48·5	24·5
Lenowitz and Graham	White	14	36	22
	,,	15	42	27
	Negro	19	45	26
	White	21	55	34
	,,	22	42	20
	,,	24	36	12
	,,	27	40	13
	,,	30	52	22
Average		21·5	43·5	22
Average of all cases		23	46	23

Schrek and Lenowitz (1947) studied 139 cases of carcinoma of the penis in white and coloured men (100 white and 39 coloured) admitted to a Veterans' Hospital in Illinois. None of them had been circumcised before the age of 6. Comparison was made with control groups of men admitted to the same hospital for other affections. The incidence of venereal disease upon the various series was the same. The results (Table V) show that circumcision in the first six years was protective, while circumcision in later years (6–35) showed no significant difference in distribution between the various groups.

Lenowitz and Graham (1946) give further details of these cases from a Veterans' Hospital which enable one to compare these data with those of Dean

TABLE V.—*Circumcision in Patients with Carcinoma of the Penis and in Control Patients.* (Schrek and Lenowitz.)

	Number of patients.	Percentage circumcised at age of—		
		0–5 years.	6–16 years.	17–35 years.
White.				
Men with carcinoma of penis	100	0	2	5
Controls	188	12·8*	2·1	3·7
Coloured.				
Men with carcinoma of penis	39	0	0	3
Controls	168	17·9*	3·0	3·6

* Difference from group with carcinoma of penis is significant.

and Lewis (Table IV). The averages of the two series are very similar, which suggests that the cases, though only 16 in number, provide a reasonable basis for study (Fig. 1). Circumcision at ages from 14 to 45 did not prevent the development of cancer after from 8 to 41 years, the mean interval being 23 years.

Cases in which the interval between circumcision and the diagnosis of cancer was less than the minimum (8 years) in Table IV have been omitted, in view of the possibility that cancer was already present at the time of the operation. Thus Lewis (1931) says of two of his cases, in which cancer was found 6 years, and 6 months, after the operation, that it was "entirely possible that both these patients had early malignancy at the time of the circumcision."

(d) *Cancer of the penis in Ceylon, Siam and China.*

This form of cancer is extremely frequent in some countries where circumcision is not practised and phimosis is common.* Spittel (1923), writing from Colombo, in Ceylon, says: "Carcinoma of the penis is another astonishingly common form of malignant disease in this country. Between June, 1911, and June, 1915, no fewer than 91 cases were operated on by me at the General Hospital, and I was but one of three surgeons. . . . Congenital phimosis is the almost invariable accompaniment."

Thomson (1921), in giving a statistical account of 13,761 operations of all kinds at the Canton Hospital, China, records 36 cases of cancer of the penis. In a private communication Dr. Thomson writes: "Cancer of the penis is relatively common. The patients are usually in their prime, and for the most part farmers without any venereal history. The condition is usually quite advanced when the patients come to the hospital and requires a radical operation." Bercovitz (1919) tabulates the records of 131 operations for cancer performed in Hainan, China; of these operations, 29 were for cancer of the penis. Jefferys and Maxwell (1910), writing of cancer, other than that of the breast, in China, say, "The other frequent forms of carcinoma which present themselves for operation are of the lips and tongue, of the penis and of the rectum, of the uterus, and in Formosa, of the

* The importance of phimosis is of course attested also by much clinical evidence from Europe and America, e.g. in the series recorded by Dean (1935) only 4 out of 93 patients had a freely retractible prepuce. The only evidence to the contrary which I have found in the literature is that of Lewis (1931) in a report on 34 cases of carcinoma of the penis operated on at Johns Hopkins Hospital; he says, "Nineteen of our series had retractible foreskins without a history of phimosis, five had acquired phimosis during the growth of the tumour, while nine gave a history of a long non-retractible foreskin." A retractible prepuce does not, of course, of itself ensure cleanliness.

mouth. . . ." They quote the words of another surgeon, McCartney, practising in China: "Epithelioma of the penis is a comparatively frequent disease, and we have made a complete amputation in a number of cases during the year . . ."

Noble (1943) records 52 cases of carcinoma of the penis (49 Siamese, 3 Chinese), with phimosis in 24 of these, in 231 admissions of males for carcinoma during 4 years at Bangkok, Siam.

Hence the available evidence is concordant in showing that cancer of the penis—

(i) is absent in those circumcised soon after birth;
(ii) occurs in those circumcised from the 3rd to the 14th year;
(iii) is very common in some countries of Asia where circumcision is not practised.

4. *Possible Modes of Action of Circumcision as a Preventive of Cancer.*

Cancer of the penis is thus a form of cancer which is dependent upon a single known factor, namely, the presence of a prepuce, for it is prevented altogether by removal of this factor at a sufficiently early age. "The prophylactic treatment of cancer of the penis consists in circumcising all male infants a few days after birth" (Dean, 1935). The postponement of the operation for 14 years only appears, on the evidence of the series of Dean, and of Lenowitz and Graham, to allow changes to occur which will later, after an interval of the order of 23 years, culminate in the development of cancer. The possible implications of this difference between early and later circumcision are so important that the evidence must be examined in detail.

(a) *Removal of the cancer-bearing area.*

One must, of course, consider the possibility that the immunity of circumcised persons from cancer of the penis is due to the removal of the area where such cancers would arise. But according to most authorities the prepuce is not the most common site of cancer. Purdy Stout (1932) says: "The vast majority of penile cancers are squamous-cell epithelioma, starting from the corona, sulcus or glans penis," *i.e.* from parts not removed in circumcision. Lederman (1941) says: "In general, it would seem that this frequency decreases in the following order—coronal sulcus, glans, prepuce and frenum; but in an appreciable number of cases the disease when first seen is too advanced to allow of such refinement of observation." Barney (1907) says that "in 65 cases, where there was any definite statement on this point 45 . . . had started on the glans, while 24 had their origin on the prepuce. . . . Many had started in the sulcus behind the corona glandis . . ." [there is a slight arithmetical error here]. Ngai (1933) states that in 89 cases of cancer of the penis in Chinamen the tumour, according to the patient, arose in the following areas:

Glans	39
Prepuce	37
Coronary sulcus	11
Frenulum and meatus	1 each
	89 cases

Leighton (1932) in a study of 67 cases says: "In 30 of our cases the disease began on the glans penis and in 13 the first appearance was on the prepuce, while a few have thought that the growth began in the sulcus." Dean (1935) in his study of 120 cases (see above) says: "In this series nearly all the early lesions grew from the proximal third of the glans, the coronal sulcus, close to either side of the frenum, or from the mucosa of the prepuce in its proximal third." In the 34 operable cases recorded by Lewis (1931) the growth was said to have begun on the glans in 20, on the sulcus or corona in 6, and on the prepuce in 4.

Föderl (1926) gives the distribution in his series from the 2nd Surgical University Clinic in Vienna as follows:

Sulcus coronalis . .	12	Frenulum . . .	3
Glans	11	Orifice of Prepuce .	3
Prepuce, internal surface	6	Orifice of Urethra .	3
Prepuce, external surface	1		

Some other authorities, however, record a larger proportion of preputial growths. Lenowitz and Graham (1946) in their study of 139 cases at the Hines Veterans' Hospital, Illinois, class together growths on the frenum and prepuce and give the following figures for those in which the site of origin could be ascertained:

	Frenum and prepuce.	Glans.	Corona.
White	38	27	4
Negro	21	7	5
Total . . .	59	34	9

Kaufmann (1886) states that out of his 33 cases, 29 began as a small wart, and of these 16 were on the prepuce and 13 on the glans. But a series of 29 cases is perhaps scarcely adequate for a statistical decision.

Thus, if one allows that as much as one-half of carcinomas of the penis arise on the prepuce, circumcision, if it acted solely by elimination of a potential cancer-bearing area, would lessen the incidence by one-half and would not account for the complete immunity of Jews. On this basis the number of cases to be expected in Sorsby's calculation (p. 336) would be 6 instead of 12.

(b) *Resistance of epithelium.*

Dean (1935) makes the following suggestion: "There must be some reason why circumcision in infancy immunizes against cancer of the penis while circumcision later in life does not. It may be that when an infant is circumcised and the glans is no longer protected by the prepuce, a denser, thicker epidermis develops, which is resistant to the formation of cancer by chronic irritation. When circumcision is performed in later years the glans may have lost its ability to produce a resistant covering, and although there is no longer irritation from retained secretions, the glans remains relatively sensitive to the contacts of everyday life."

(c) *External and internal agents.*

This suggestion brings one to the important question whether cancer of the penis is caused by a wholly external agent, in the same way as cancer of the scrotum can be caused by soot, or by an agent arising from the body, either

between the prepuce and glans, or deep to the epidermis. A purely external agent appears unlikely, for a circumcised person is exposed, not less, but more, to such a factor, and the thickened epidermis supposed by Dean would have to compensate for such exposure; moreover an unretracted prepuce, the usual antecedent of cancer, would be protective. The influence of phimosis in predisposing to cancer of the penis suggests that the carcinogenic agent is formed in material between the prepuce and glans.

DISCUSSION.

Perhaps the most probable, and the most interesting, interpretation of the data given above is to suppose that the train of events leading to this malignant growth is set going in the earlier years of life, and that removal of the cause does not then avert the final appearance of cancer at a much later age. Thus, even if we ignore all the evidence from Moslem circumcision in view of any doubt about the completeness of the operation, the data collected by Dean at the Memorial Hospital, New York, and by Schrek and Lenowitz at a Veterans' Hospital in Illinois (Tables IV and V, Fig. 1), show that circumcision at the age of 14 and onwards may be followed by the development of cancer after an average interval of 23 years, while circumcision in infancy prevents this disease altogether. This may hold good of other forms of cancer also. Schrek and Lenowitz, in their study of cancer of the penis, suggest that "Perhaps other factors operating in infancy determine whether the adult develops other types of cancer." Thus cancer of, say, the stomach, arising after the second 25 years of life, may be predestined to occur by factors to which the body was exposed during the first 25 years after birth. This idea suggests interesting possibilities of the prophylaxis of cancer by attention to the hygiene of youth. The juvenile death-rate, as a measure of social factors, draws attention to the children who die, whereas those who do not die are, from the present point of view, more important; a child may survive injury by social conditions of which the effect appears later in life.

The mean age at diagnosis of the 100 white men studied by Schrek and Lenowitz (Table V) was 50·4, while the corresponding age of the seven among them who had been circumcised was 43.* Admittedly these numbers are small for any comparison of averages, but the earlier age of the seven suggests that the operation had no protective action signalized by postponement of the disease. This indication is compatible with the idea suggested here that the occurrence of this form of cancer is determined at an early age. Possibly the earlier development of cancer in the circumcised men was due to the carcinogenic effect of phimosis, for which the operation was performed.

This phenomena of the appearance of cancer long after the cessation of exposure to a causal agent is, of course, known to occur in the case of industrial cancers, *e.g.* cancer of the scrotum may arise in a cotton spinner many years (from 1 to 14, Henry (1928)) after he has left the mule room. X-ray cancer provides a good instance of the same process and is of peculiar value, because the cessation of the primary action is abrupt, whereas chemical agents may persist for an unknown length of time in the skin. But the instance of cancer of the penis is of more interest, first, because no artificial agents are concerned, and second, because the essential changes take place in the early years of life.

* No comparison is possible with the age of maximum incidence in the general population, which is later, as the Veterans are a group selected as regards age.

SUMMARY.

1. Cancer of the penis does not occur after circumcision on the eighth day according to the Jewish practice, but occurs in later life in Moslem populations, where the operation is carried out between the 3rd and 14th years. But no record has been found in the literature of the age-relations in even a single case of cancer of the penis in a Moslem.

2. Sixteen recorded cases of cancer of the penis following surgical circumcision at a mean age of 23 (range 14 to 45) developed cancer of the penis after a mean interval of 23 years (range 8 to 41 years).

3. The failure of the operation deferred until the 14th year to give the protection given by it when carried out on the 8th day suggests that the train of events leading to the malignant growth is set going early in life, and that removal of the cause does not then avert the development of cancer at a much later age. Other forms of cancer are perhaps due to factors acting in youth.

4. Cancer of the penis is very prevalent among some peoples of Asia who do not practise circumcision.

5. The protection given by the Jewish operation is not due to removal of the cancer-bearing area.

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THE FREQUENCIES OF LEFT- AND RIGHT-SIDED BREAST CANCER.

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From the Danish Cancer Registry under The National Anti-Cancer League.

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BECAUSE of the anatomical and physiological symmetry of the breasts it is generally assumed that also their pathological symmetry goes without saying—nay even without documentation.

The question whether breast cancer arises with the same frequency in both breasts became topical to one of us while examining whether relatives with breast cancer were more likely than other persons to develop their tumours of that organ on the same side.

For our examination we have used the material of hospital cases notified to The Danish Cancer Registry. We are indebted to our medical colleagues for their interest, and the result that out of 4139 female cases of carcinoma from the years 1942–46 inclusive, only 17 have not been referred to any of the two sides.

The total Danish material will naturally also include cases from private practice known to the Cancer Registry only through death certificates. Because of the difficulties involved in obtaining accurate information as to the site of the tumour in many of these cases they have been left out of the present examination. They amount to about 130 cases each year. Thus 412 cases from the years 1942–44, known only from death certificates, consisted of 81 left-sided and 64 right-sided cases, together with 7 bilateral and 260 unstated cases.

Table I gives the number of cases for each of the two sides, distributed according to age. Similar tables showing the numbers of unmarried, married, divorced and widowed persons with breast cancer have also been worked out to examine if these conditions might influence the side of the tumour. The table gives these figures for the whole period, and for each year. The sum total shows 2117 tumours of the left and 1908 of the right breast. In 97 cases tumours were stated to have occurred on both sides, while the localization of 17 tumours was unknown.

The question now arises if this surplus of 209 cases in the left breast is statistically significant or may be due to chance.

In order to test the hypothesis that left- and right-sided breast cancers occur with equal frequency, the following considerations were made:

Consider n observations of the same kind, by which the events A_1, A_2, A_3 occur with the probabilities p_1, p_2, p_3 respectively, where $p_1 + p_2 + p_3 = 1$. The probability that A_1, A_2, A_3 will occur a_1, a_2, a_3 times respectively is then according to the multinomial theorem:

$$p\left\{a_1, a_2, a_3\right\} = \frac{n!}{a_1!\, a_2!\, a_3!}\; p_1^{a_1}\, p_2^{a_2}\, p_3^{a_3}, \text{ where } a_1 + a_2 + a_3 = n.$$

If we put $p_1 = p_2 = p$ we get

$$p\left\{a_1, a_2, a_3\right\} = \frac{n!}{a_1!\, a_2!\, a_3!}\, p^{a_1 + a_2}\, (1 - 2p)^{a_3},$$

TABLE I.—*Cancer of Left and Right Breast.*

Age.	1942.					1943.					1944.				
	L.	R.	Bilateral.	Unsp.	Total.	L.	R.	Bilateral.	Unsp.	Total.	L.	R.	Bilateral.	Unsp.	Total.
15–19															
20–24	1	2			3										
25–29			1		1	2				2	1	2			3
30–34	6	9	1		16	7	8			15	16	10	2		28
35–39	14	14	1		29	21	18		1	40	20	24			44
40–44	33	33	5		71	40	42		1	83	42	37	2		81
45–49	49	39	6		94	60	53	4		117	55	50	3		108
50–54	42	34	1		77	32	45	4		81	51	34	2		87
55–59	63	44	4		111	68	48	2		118	60	44	3	2	109
60–64	49	44	4	1	98	65	60	2	1	128	63	43	2		108
65–69	43	39	3		85	50	37	7		94	80	45	4		129
70–74	29	28	2		59	35	37	1		73	40	33	3		76
75–79	11	14	1		26	22	20	3	1	46	20	20	2		42
80–84	5	5			10	9	10	2	1	22	11	9			20
85–89	3	2			5	1	1	1		3	4	5			9
90–94															2
unstated														1	1
Total	348	307	29	1	685	412	379	26	5	822	464	357	23	3	847
Of which unmarried	65	57	6	1	129	82	73	7		162	95	77	12		184

Age.	1945.					1946.					1942–46.				
	L.	R.	Bilateral.	Unsp.	Total.	L.	R.	Bilateral.	Unsp.	Total.	L.	R.	Bilateral.	Unsp.	Total.
15–19							2			2		2			2
20–24		1			1	1				1	2	3			5
25–29	4	2			6	4	1			5	11	5	1		17
30–34	13	13			26	7	7			14	49	47	3		99
35–39	21	20			41	32	28			60	108	104	1	1	214
40–44	49	34			83	46	40			86	210	186	7	1	404
45–49	52	44	2		98	65	60	2	1	128	281	246	17	1	545
50–54	59	62	1		122	56	56			112	240	231	8		479
55–59	63	52	3	1	119	59	60			119	313	248	12	3	576
60–64	55	69			124	58	51	2		111	290	267	10	2	569
65–69	51	63	1	2	117	53	43	6	1	103	277	227	21	3	528
70–74	36	47		2	85	29	37			66	169	182	6	2	359
75–79	15	19			34	28	26			54	96	99	6	1	202
80–84	10	14			24	12	8	2	1	23	47	46	4	2	99
85–89	6	2			8	8	4			12	22	14	1		37
90–94	1				1						2	1			3
unstated														1	1
Total	435	442	7	5	889	458	423	12	3	896	2117	1908	97	17	4139
Of which unmarried	86	94	1		181	92	96	2	1	191					

TABLE I—*continued.*

Unmarried women 1942–46.

	L.	R.	Both	Unsp.	Total.
15–19		2			2
20–24	2				2
25–29	1	1			2
30–34	10	6	2		18
35–39	19	13			32
40–44	51	50	2		103
45–49	50	65	4		119
50–54	37	49	4		90
55–59	61	56	3		120
60–64	63	54	3	1	121
65–69	52	47	4		103
70–74	39	33	3		75
75–79	18	15	2		35
80–84	13	5		1	19
85–89	4	1	1		6
90–94					
unstated					
Total	420	397	28	2	847

and by the binomial theorem we get for the probability of $(a_1 + a_2)$ events of either of the two kinds A_1 and A_2 in n observations :

$$p\left\{a_1 + a_2\right\} = \frac{n!}{(a_1 + a_2)!\, a_3!}\, (2p)^{a_1 + a_2}\, (1 - 2p)^{a_3}.$$

The probability that A_1 occurs just a_1 times in a series of n observations where just $(a_1 + a_2)$ of the results belonged to either of the groups A_1 and and A_2 is then

$$p\left\{a_1, a_2, a_3 \,\middle|\, a_1 + a_2\right\} = \frac{(a_1 + a_2)!}{a_1!\, a_2!}\, (\tfrac{1}{2})^{a_1 + a_2} \qquad (1)$$

This result is identical with the one obtained if n_1 observations are made with the probabilities $p_1 = \frac{1}{2}$ of the event A_1 and $p_2 = \frac{1}{2}$ of A_2 and the probability of getting the event A_1 just a_1 times is sought :

$$p\left\{a_1\right\} = \binom{n_1}{a_1}\left(\frac{1}{2}\right)^{n_1}$$

as we get the right side of (1) if we put $n_1 = a_1 + a_2 = n - a_3$.

The procedure involved in (1) : by n observations $(n = a_1 + a_2 + a_3)$ to keep $(a_1 + a_2)$ at a certain fixed value is then tantamount to omitting the a_3 cases of the event A_3 and confining the considerations to the remaining $n - a_3$ observations in testing the hypothesis that A_1 and A_2 are equally frequent.

As an approximative test it can be asserted that the quantity

$$u = \frac{\frac{a_1}{n_1} - \frac{1}{2}}{\sqrt{\frac{1}{2}\cdot\frac{1}{2}\cdot\frac{1}{n_1}}} = \sqrt{\frac{a_1 - a_2}{n_1}}$$

approximately should be normally distributed with mean zero and variance 1, if $p_1 = p_2$.

For the application to the problems in question we interpret :

A_1 = tumour in the left breast ;
A_2 = tumour in the right breast ;
A_3 = bilateral or unstated localization of breast cancer ;

and calculations then give $u = 3{\cdot}29$, corresponding to a value P of about 1 per thousand.

Assuming that the left and right breast cancer was equally frequent, calculations according to the tests described have shown that only one out of a thousand samples of the size of the present data could be expected to show a deviation equal to, or exceeding the differences observed. Thus the preponderance of left-sided breast cancer can be considered as statistically significant, and according to the present material each hundred cases of cancer in the right breast will correspond to 111 of the left.

In working out the material we found some variations in the left-side preponderance of the different years. Especially the year 1945 showed a slightly

higher frequency of right-sided tumours, and computations were therefore carried out to ascertain whether these variations would contradict the assumption of a constant inequality with regard to lateral distribution of breast cancers in all five years.

TABLE II.—*Number of Left-sided Breast Cancers per Thousand Cases with Known Localization.*

Year.	Married women, etc.	Unmarried women.	Total.
1942	531	533	531
1943	519	529	521
1944	569	552	565
1945	501	478	496
1946	528	489	520
1942–46	529	514	526
χ^2	6·59	2·73	8·50
f	4	4	4
P	>0·10	>0·50	>0·05

The χ^2 test carried out in Table II gives the value 8·50 with four degrees of freedom, and as this corresponds to $P>5$ per cent, it means that the material does not disprove the assumption of constant inequality in lateral distribution.

Considering the well-known differences in frequency of breast cancer among married and unmarried women, we have tested the corresponding values for breast cancer of each side for two groups, respectively of women not previously married, and of the remaining group of married, divorced and widowed women. We found for each hundred right-sided cancers, 105·8 left-sided tumours among unmarried, and 112·3 among the remaining women, and this difference between the two groups cannot be considered as significant.

TABLE III.—*Number of Left-sided Breast Cancers per Thousand Cases with Known Localization.*

Age.	Married, divorced and widowed women.						Unmarried women 1942–46.	Total 1942–46.
	1942.	1943.	1944.	1945.	1946.	1942–46.		
34	333	539	591	483	556	505	591	521
35–39	462	514	436	486	556	494	594	509
40–44	480	500	517	623	567	539	505	530
45–49	580	548	566	559	556	561	435	533
50–54	574	438	614	505	517	527	430	510
55–59	609	554	610	574	500	568	521	558
60–64	506	510	588	455	537	516	538	521
65–69	533	586	660	449	530	556	525	550
70–74	468	466	542	406	451	466	542	482
75–79	476	525	433	440	500	482	545	492
80–	538	500	423	464	583	495	739	538
All ages	531	519	569	501	528	529	514	526
χ^2	7·91	4·53	12·94	11·25	2·88	13·38	12·05	9·69
f	10	10	10	10	10	10	10	10
P	>0·50	>0·90	>0·20	>0·30	>0·98	>0·20	>0·20	>0·50

In order to examine whether the higher frequency of left-sided breast cancer varies with age, Table III has been worked out. χ^2 tests, however, revealed no significant variations.

Searching for similar materials from other countries we found a report on the Swiss cancer census from the years 1933–35 by Fellenberg (1940), giving the figures for left- and right-sided breast cancer. The total amounted to 1045 cases, of which 522 were localized to the left and 464 to the right breast; 22 cases were bilateral and 37 without statement of side.

Computations carried out according to the methods described give a value for u of 1·85, so that, even if no statistically significant difference has been demonstrated, the tendency is nevertheless in agreement with the findings from the Danish material, and of almost the same order of magnitude. Thus for each 100 cases of cancer in the right breast we find in Denmark 111 left-sided, and in Switzerland 113 left-sided tumours.

It is outstanding that out of the total surplus of 58 left-sided cases, 50 are found in the material from Zurich, but the application of the χ^2 test shows that this may be due to chance alone.

Our attention has been called to the fact that in his Statistical Review for 1935 (p. 86) the Registrar-General of England and Wales analysed the deaths from breast cancer in 1931–34 as follows :—

	R.	*L.*	*Bilateral.*	*Unspecified.*
Males	30	33	—	7
Females	2623	2995	643	269

and commented that cancer of the left breast was more frequently certified than cancer of the right breast to the extent of 14 per cent. The proportion of bilateral cases is much greater than in the Danish material, as might be expected, since they consist only of fatal cancers. If *total* occurrences in right and left breasts are compared after adding the bilateral cases to the numbers for each side, the left-sided excess becomes 11 per cent, which is the same as the author's find from the Danish data.

Table IV quotes the report of Janet E. Lane-Claypon (1926), examining the effect of injury to the breast on the frequency of cancers respectively of the same and the opposite breast.

TABLE IV.—*Figures According to Janet Lane-Claypon.*

Type of injury to the breast.	Tumour in the—	Number of cases with injury to—				Computed value of u.
		Left breast.	Right breast.	Both sides.	Side unstated.	
Injury and bruising	Same breast	29	12	..	..	2·66
,, ,,	Opposite breast	1	1	..	..	..
Injury, no bruising	Same ,,	50	39	..	..	..
Injury doubtful	,, ,,	2	4	..	..	..
Slight injury	,, ,,	20	21	..	..	..
Injury and bruising	No tumour	5	2	1	2	..
Injury, no bruising	,,	7	6	..	5	..
Slight injury	,,	2	1	..	2	..
Total (slight inj. excl.)	..	92	60	1	7	2·60

It is seen that in this material also the left breast shows more cancers than the right. However, concerning the coincidence of injury with cancer, the figures are too small to permit any direct comparisons of the two sides.

Application of the tests described gives the result that injuries seem to occur more frequently to the left than to the right breast. This applies both to the cancer patients separately and to the total material, and thus it follows that cancers coinciding with injury are more frequent in the left than in the right breast.

Considering the pretexts for the development of cancer in the two breasts, it does not seem unreasonable to suppose that the different incidence demonstrated may be connected with difference in use of the upper extremities, for instance by lactation or by protection against injury. But whether the differences in injuries sustained by the breasts has any connection with the difference in cancer incidence, or is a mere coincidence, nobody can tell at present.

SUMMARY.

Among 4139 female cases of breast cancer notified from hospitals to The Danish Cancer Registry, 2117 tumours were localized to the left and 1908 to the right breast, while 97 were bilateral and 17 without statement of side.

Applying a special test the authors demonstrated that, provided cancer frequency of the two breasts were the same, only one out of a thousand samples of the size investigated could be expected to show a deviation equal to, or exceeding the difference observed.

Smaller materials from Switzerland, 1935, and England, 1926, show similar features. Thus for each 100 cases of cancer in the right breast we find in Denmark 111 left-sided, and in Switzerland 113 left-sided tumours. The English material shows a higher frequency of injury to the left than to the right breast.

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THE SKIN LESIONS OF MONOCYTIC LEUKAEMIA.

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SINCE 1913, when Reschad and Schilling-Torgau (1913) first distinguished monocytic leukaemia from the lymphatic and myelogenous types, over 250 cases have been reported (Evans, 1942). In their case report Reschad and Schilling-Torgau noted widely distributed nodules in the skin, which, histologically, were packed full of cells similar to those present in the peripheral blood. In most instances subsequent observers have focused their attention on the interesting haematology of the disease, whilst little attention has been paid to the relatively common and often distinctive cutaneous lesions.

In lymphatic and myelogenous leukaemias petechiae in the skin are fairly common. They usually occur in the later stages of the disease, and are indistinguishable from petechiae of other origin. Specific cutaneous changes are rare. In monocytic leukaemia, on the other hand, non-purpuric skin lesions were reported in 36 per cent of patients by Isaacs and Sturgis (1936) and in 10 per cent by Freeman and Koletsky (1939), while of 6 cases of the disease admitted to the Middlesex Hospital in the past 10 years, 2 developed non-purpuric rashes. Further, the purpura of monocytic leukaemia did not always remain petechial. In many reported cases it became raised and infiltrative, and progressed to fully developed nodular or ulcerative lesions. It would appear that the presence of such rashes should rank in diagnostic importance with ulcerative hypertrophic gingivitis. Especially interesting are the rare cases, such as the one reported below, in which a " disseminated furunculosis " or other cutaneous lesion preceded the onset of a leukaemic haematology. The possibility then arises that the leukaemic process has originated in the reticulo-endothelial cells of the skin, and has only later involved locations more intimately concerned with haemopoiesis.

Case Report.

M. F—, male, aged 20. In June, 1945, the patient first suffered an attack of furunculosis involving mainly the shoulders and neck. This gradually subsided during a 6-weeks' stay in hospital. In September, 1945, he joined the Royal Artillery as a gunner, and soon after enlistment had another attack of boils on his back, and to a lesser extent on the neck and face. As before, the lesions gradually healed during a further 8 weeks' hospitalization. In November, 1945, he was drafted to India, where he was stationed at Madras, and later at Bangalore. A few weeks after his arrival in India crops of boils began to appear once more on the shoulders, neck and face. By February, 1946, nodules had developed on the right arm. These never became pustular, but progressed to a brawny thickening, and later formed shallow ulcers. At this time he had a remittent pyrexia and his spleen was observed to be palpable. A swab taken from the

ulcers grew numerous colonies of *Staphylococcus aureus*. The leucocyte count was raised to 20,000/cu. mm. with a predominance of neutrophil granulocytes. In consequence a tentative diagnosis of staphylococcal septicaemia was made. However, all attempts to culture an organism from the peripheral blood failed. Moreover, the patient made no response to an adequate course of treatment with local and systemic penicillin.

In successive blood examinations the total leucocyte count rose, and the percentage of mononuclear cells steadily increased. The patient was repeatedly transfused with whole blood, but with no improvement in his condition. In May, 1946, he was returned to England. By this time he was an obvious case of monocytic leukaemia. The ulcers on his arm had become large and irregular, but remained shallow. They were thought to be a type of tropical ulceration. On July 21, 1946, he was transferred to the Middlesex Hospital.

On admission the patient was a pale, wasted youth. The skin of his face, shoulders and neck bore the scars of previous furunculosis. The skin over both tibiae was discoloured by almost confluent petechiae, which were beginning to fade. On the right arm there were a number of brawny, dusky, reddish-brown nodules with a somewhat metallic sheen. These ranged in size from 1–15 cm. in diameter, and the larger of them had undergone a superficial ulceration. The edge of the ulcers was raised, everted, irregular in outline, and paler than the rest of the nodule, the floor being occupied by slough covered by tenacious, purulent exudate (Fig. 1). The ulcers were extremely painful and tender on palpation. Striae atrophicae were present on the flanks and lower back. The gums were moderately hypertrophied and ulcerated, but bled only slightly. The tonsils were very large and pale. A few petechiae were seen on the buccal mucosa. Small, rubbery, mobile lymph glands filled both anterior triangles of the neck, and a few were found in the axillae and groins. The pulse rate was 100/min., and the temperature fluctuated between 99 and 101° F. A soft systolic murmur was heard in the pulmonary area. The spleen was palpable 5 cm. below the costal margin, and the firm liver edge was felt 7 cm. below the right rib margin. The sternum was very tender to percussion. The patient complained of some pain over the left wrist, and on palpation a tender thickening was felt overlying the dorsum of the lower end of the radius.

Blood count (27..vii.46).—Haemoglobin 43 per cent = 6·2 g/100 c.c.; erythrocytes 2·24 millions/cu. mm.; leucocyte count 277,000/cu. mm.

	Per cent.	Total/cu. mm.
Differential count:		
Neutrophil polymorphonuclears	5	13,800
Lymphocytes	1	2,700
Monocytes	90	250,000
Metamyelocytes	2	5,500
Myelocytes	2	5,500

Blood platelets 24,000/cu. mm.

Film appearances (Fig. 2): The predominant white cell was a typical monocyte, and many showed grooving of the nucleus. There were scanty peroxidase-positive granules in the cytoplasm. Sternal marrow puncture (27.vii.46) (Fig. 3): Nucleated cell count 342,000/cu.mm. (normal 25,000–100,000).

	Per cent.	Normal range %.
Differential count :		
Neutrophil polymorphonuclears	4	(20–50)
Metamyelocytes	1·5	(4–12)
Myelocytes	2·5	(2–8)
Premyelocytes	1	(0·5–5)
Lymphocytes	0·5	(5–20)
Monocytes	88	(0–5)
Monoblasts	1	—
Late normoblasts	0·5	(7–19)
Intermediate and early normoblasts	0·5	(4–15)
Pro-erythroblasts	0·25	(0–4)
Haemocytoblasts	0·25	(0–1)

A scraping from the edge of an ulcer showed the following cytology (Fig. 4) : Neutrophil polymorphonuclears 4 per cent, lymphocytes 10 per cent, monocytes 81 per cent, monoblasts 4 per cent, plasma cells 1 per cent, and occasional reticulum cells. The monocytes were typical in form and showed scanty peroxidase granules. No leishmaniae, fungi, spirochaetes, actinomycetes, or acid-fast bacilli were seen. A swab from the ulcers showed Gram positive and negative bacilli, and Gram positive cocci. On culture a heavy growth of *Ps. pyocyanea* and a moderate growth of β-haemolytic streptococci and *C. xerosis* was obtained. Wassermann reaction negative. Paul-Bunnell reaction negative to 1/7 (method of Davidsohn).

Radiograms of the left wrist showed early periosteal erosion of the dorsum of the lower end of the radius.

Progress.—The ulcers were at first dressed with hypertonic sodium sulphate containing 1 per cent β-phenoxyethanol (Phenoxetol) and later with penicillin cream without any substantial improvement. On July 30, 1946, the patient complained of some abdominal pain, and vomited all he ate, together with a little altered blood. Throughout the next day he vomited bright and altered blood. He became progressively more exhausted and died towards evening.

DESCRIPTION OF PLATES.

FIG. 1.—Photograph of the lesions on the arm, showing nodules and large serpiginous ulcers with raised, rolled edge and sloughing base.

FIG. 2.—Photomicrograph of a blood smear, showing typical cells of the monocyte series, some with grooving of the nuclei. Leishman stain. × 1440.

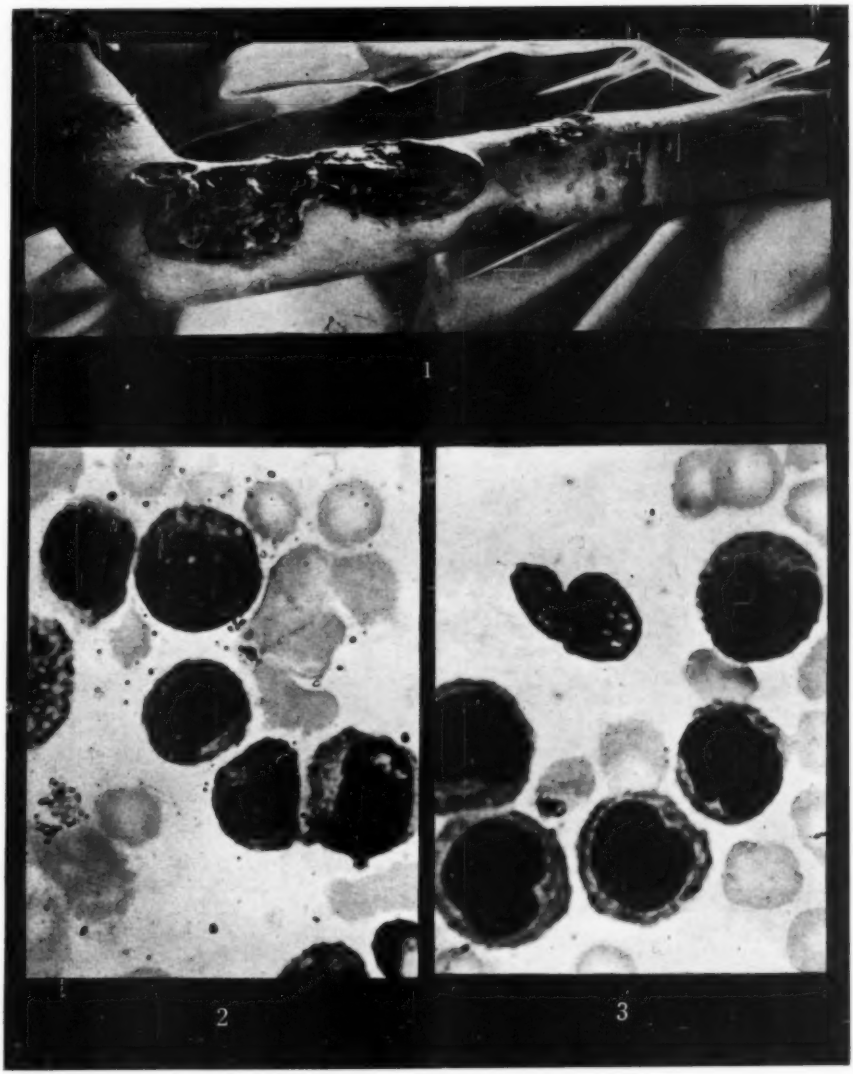
FIG. 3.—Photomicrograph of a sternal marrow smear. Grooved monocytes and promonocytes are seen. Leishman stain. × 1440.

FIG. 4.—Photomicrograph of a scraping from the edge of a skin ulcer, showing grooved monocytes, one of which has a pseudopodium. Leishman stain. × 1440.

FIG. 5.—Photomicrograph of a section of the periphery of an ulcer on the arm, showing normal epithelium with a heavily infiltrated dermis, passing into an area of hyaline necrosis with intense monocytic infiltration and overlying epithelial degeneration. Haematoxylin and eosin. × 17.

FIG. 6.—Photomicrograph of a quiescent "furuncle" showing a central zone of hyaline necrosis with heavy infiltration at its edge. In the surrounding region there is a light monocytic infiltration mainly around blood vessels and hair follicles. Haematoxylin and eosin. × 17.

FIG. 7.—Photomicrograph of rectangular area in Fig. 6 showing the infiltration of monocytic cells around the hair follicles and sweat glands. × 125.



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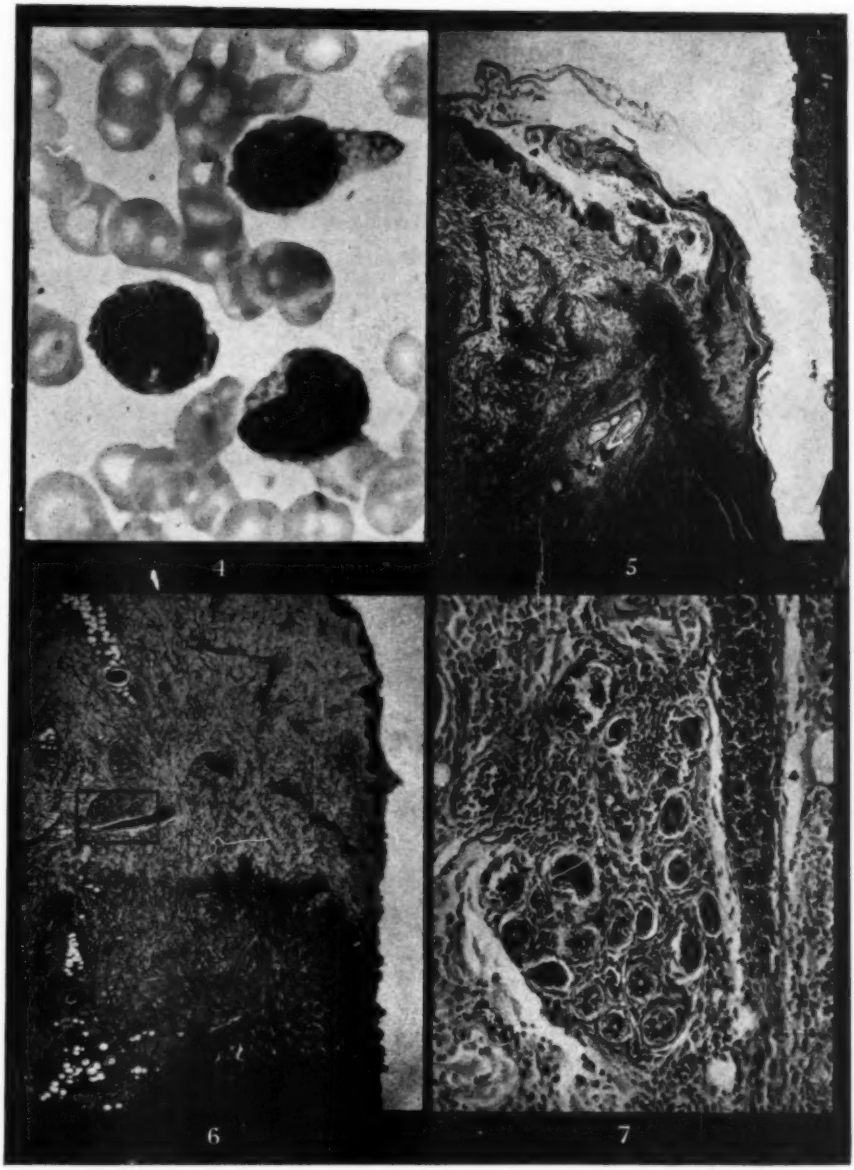

Fairburn and Burgen.

Autopsy findings.

The mouth showed early interdental papillary ulceration. There was marked hypertrophy of the faucial, lingual and naso-pharyngeal tonsils. All the serous membranes showed multiple petechiae and contained varying amounts of blood-stained fluid. There was moderate dilation of the heart, and the myocardium showed early fatty change. The lungs contained multiple recent infarcts up to 4 cm. in diameter. The stomach and intestines were full of altered blood, and the mucosa exhibited many dusky nodular infiltrations up to 1 cm. in diameter. Many of these were superficially ulcerated. The liver weighed 2700 g.; its cut surface was soft and pale, but the pattern was still discernible. The spleen weighed 1350 g., was pale, and had completely lost its pattern. The lymph glands throughout the body showed moderate discrete enlargement. The cut surfaces were greyish-yellow and homogeneous but for areas of recent haemorrhage. The femoral bone-marrow was a uniform greyish-pink hue throughout. Sub-periosteal leukaemic deposits were found in the popliteal region of the right femur and the distal parts of the left ulna and radius.

Histology.—The nodular areas showed a heavy infiltration of the entire dermis and superficial subcutaneous tissues with monocytic cells with patchy hyaline eosinophil necrosis and ulceration. The vessels were packed with monocytes (Fig. 5). A section of a subsiding furuncle showed a dense invasion with mononuclear cells, while in the less heavily involved edge the hair follicles and sweat glands alone were infiltrated by similar cells. (Figs. 6 and 7). The sternal and femoral bone marrow was extensively replaced by monocytic cells, which were comparatively large and round, oval or pyriform in shape. The cytoplasm was ill-defined. The nucleus was oval, reniform or lobular; it was finely granular with occasional hyperchromatic bars. A distinct nuclear membrane could be seen. There were a moderate number of mitoses but no nucleoli. The lymph glands and spleen had lost their normal architecture, and were diffusely infiltrated with cells of the monocytic series. The liver showed a uniform necrosis of the mid and central zones of the lobule with a heavy peripheral infiltration. The nodules in the intestine were uniformly packed with monocytes.

DISCUSSION.

A large variety of skin lesions have been described in association with monocytic leukaemia. Most of these are specific in the sense that histological examination of the skin reveals an infiltration, or the local production of cells of the monocytic series. It has been claimed, however, that there is a "toxic" group of lesions (or leukaemides), including haemorrhagic, urticarial, bullous, eczematous and exfoliative types, presumably showing no extravascular monocytic cells. That most of these lesions are really infiltrative has been established by biopsy on repeated occasions (Whitby and Christie, 1935; Montgomery and Watkins, 1937).

We have collected the reports of 50 cases of undoubted Schilling-type monocytic leukaemia in which cutaneous lesions were noticed at some time during the course of the disease (Table I). These are mainly derived from cases published in the English language, but otherwise show no bias. When the skin lesions were of relatively long duration it has sometimes been difficult to believe that any

TABLE I.

Authors and date.		Age.	Sex.	Duration.	Rash.	Distribution.
Reschad and Schilling-Torgau	1913	33	M	9/52	Purpura; maculo-papular and nodular	Generalized.
Dameshek	1930	55	M	4/12	Maculo-papular	Generalized.
Lawrence and Josey	1931	13	M	1/12	Purpura	,,
Asselstine	1932	35	M	5/12	,,	,,
Clough	1932	62	M	9/12	,,	Trunk
Farrar and Cameron	1932	55	M	7/12	,,	,,
Gardner	1932	47	F	3/12	,,	Generalized.
Sydenstricker and Phinizy	1932	47	F	5/12	1, Purpura; 2, macules; 3, plaques; 4, furuncles	1, Generalized; 2, trunk and lower limbs; 3, trunk and lower limbs; 4, trunk.
Foord, Parsons and Butt	1933 1.	17	M	7/52	Purpura	Trunk.
	2.	22	F	3/12	,,	,, upper and lower limbs.
Fowler	1933	43	M	5/12	Pustules, macules, plaques	,, face and neck.
Orr	1933	40	M	18/12	Pustules	Generalized.
Doan and Wiseman	1934	5	M	3/12	Purpura, nodules	,,
Forkner	1934 1.	31	M	3/12	Purpura	Trunk, upper and lower limbs.
	2.	38	M	5/12	,,	,, ,, ,,
	3.	40	M	11/52	,,	,, ,, ,,
	4.	16	F	5/12	,,	,, ,, ,,
	5.	32	M	11/52	,,	,,
Gregg	1934	58	M	4/12	Furuncular	Face, upper limb.
Labbé, Boulin, and Balmus	1934	29	M	2/12	Pustules	Generalized.
Levine	1934 1.	28	M	6/52	Purpura	Upper and lower limbs.
	2.	41	M	3/52	,,	Trunk.
	3.	68	F	4/12	,,	Generalized.
Morgan and Hsu	1934	34	M	1/12	1, Purpura; 2, plaques	1, Generalized; 2, trunk.
Mann	1935	57	M	5/52	Nodules, plaques	Face, neck and upper limbs.
Mercer	1935 1.	39	M	2/12	Macular, papulo-nodular	Generalized.
	2.	47	F	3/12	1, Maculo-papular; 2, nodular	1, Trunk and lower limbs; 2, trunk.
Mitchell	1935	63	M	8/12	1, Purpura; 2, maculo-papular; 3, nodular	1, Generalized; 2, lower limbs; 3, trunk, upper and lower limbs.

Whitby and Christie	1935		44	F	10/12	Purpura	Generalized.
Klump and Evans	1936		56	M	1/12	1, Suppurating ulcers; 2, purpura	1, Lower limbs; 2, generalized.
Loveman	1936		31	F	1/12	Papules, nodules	Trunk, lower limbs.
Lynch	1936		67	M	5/52	Purpura	Generalized.
Montgomery and Watkins	1937	1.	68	M	6/12	„	„
		2.	62	M	4 yrs.	Chronic eczematous lesions	„
		3.	29	F	1 yr.	Exfoliative dermatitis	„
Osgood	1937	1.	7	F	1/12	Purpura	Trunk.
		2.	36	F	7/12	1, Purpura; 2, maculo-papular	1, Lower limbs; 2, face, neck, upper and lower limbs.
		3.	62	M	2 yrs.	1, Furuncles; 2, carbuncle	1, Generalized; 2, neck.
		4.	76	F	18 days	Purpura	Lower limbs.
		5.	67	M	1 yr.	„	Generalized.
Montgomery and Watkins	1938	1.	20	M	4/12	Exfoliative dermatitis	„
		2.	76	M	17/12	1, Exfoliative dermatitis; 2, plaques; 3, nodules; 4, bullae; 5, pigmentation; 6, lichenification; 7. impetigenous lesions	1, Generalized; 2, lower limbs; 3, lower limbs; 4, hands and feet; 5, generalized; 6, lower limbs; 7, face.
		3.	58	M	6/12	Exfoliative dermatitis	Generalized.
Freeman and Koletsky	1939		78	F	16/12	Purpura, macules, papules, nodules, plaques, bullae	Trunk, upper and lower limbs.
Lamb and Stout	1940		72	M	14/12	1, Papules; 2, macules; 3, vesicles and bullae; 4, exfoliative dermatitis; 5, pigmentation; 6, lichenification; 7, nodules; 8, impetigenous lesions	1, Upper limbs; 2, face, neck, upper limbs; 3, upper limbs; 4, generalized; 5, generalized; 6, lower limbs; 7, lower limbs; 8, face.
Gueft	1943		2/12	—	1 yr.	Papulo-nodular	Trunk, upper and lower limbs.
Biggart	1945		20	F	11/52	„	„
Wechsler	1945		29	M	6/52	Purpura	„
Herbut and Miller	1947	1.	16	M	26 days	Papules, purpura	Generalized.
		2.	57	M	1 yr.	1, Furuncles; 2, purpura	1, Face, neck, trunk; 2, generalized.

but a fortuitous association has occurred between the rash and the disease. These cases have been excluded from our series.

We have classified the lesions into six groups: (1) Purpuric, (2) Maculopapular, (3) Plaques and nodules, (4) Cutaneous suppurative conditions, (5) Exfoliative dermatitis, (6) Miscellaneous.

Group 1: *Purpura.*

The purpuric lesions are usually very widespread, and more often petechial than ecchymotic. Occasionally the petechiae develop into papules or other types of rash, but more frequently when several lesions coexist the purpura is preterminal.

Group 2: *Macules and papules.*

These lesions are more or less generalized, but usually asymmetrical in distribution. Typically a roseolar maculo-papular eruption appears, closely simulating an early secondary syphilide. Changes in character from day to day, often of a cyclical nature, may occur. Minute red dots and fine bluish lines are not infrequently seen in these lesions, and later, after the erythema has subsided, slightly indurated slate-blue areas may remain (Mercer, 1935). Finally, if involution does occur the rash often leaves in its wake faint grey taches imparting a mottled appearance to the skin. Although the lesions are sometimes tender on pressure, pruritus or pain is rare. The rash does not usually precede other symptoms of leukaemia.

Group 3: *Nodules and plaques.*

Usually these are pale and shot-like, and situated deeper in the skin than the previous group, being more easily felt than seen. On occasion, they enlarge to become plaques. At this stage they may be difficult to distinguish from mycosis fungoides (Montgomery and Watkins, 1937) or cutaneous sarcoidosis. The centres sometimes soften and slough, leaving irregular crateriform ulcers with an indurated base and a raised infiltrated edge. There is usually some degree of secondary infection in these cases. All grades of transition between Group 2 and 3 lesions may occur.

Group 4: *Cutaneous suppurative conditions.*

This group contains those lesions which appear clinically to be primary suppurative affections of the skin. All grades of suppurative lesion may be found, from small pustules and furuncles to carbuncles. They may be generally distributed, appear as an impetigo contagiosa on the face, or mimic an acne vulgaris on the face, neck and back (as in the case reported here). Not infrequently these lesions precede by many months all other manifestations of monocytic leukaemia. The natural history is varied. Resolution may occur, often leaving a residual brown pigmentation; indurated, violaceous papules may appear around the primary lesion; or sloughing may give rise to indolent ulcers. Fresh crops of pustules may appear from time to time.

Group 5 : Exfoliative dermatitis.

Chronic eczematoid areas, lichenification, and plaques simulating early mycosis fungoides may precede or be associated with exfoliation (Montgomery and Watkins, 1938*a* and *b*). In some cases the skin condition preceded other leukaemic changes by months or years. In these instances it is very difficult to assess a causal relationship to the leukaemia. Montgomery and Watkins (1938*a*) quote the case of a patient who had suffered from chronic eczema for many years, and later developed an exfoliative dermatitis which was proved by biopsy to be leukaemic. There must be an element of doubt in such cases unless skin biopsy performed at an early date has shown the characteristic picture of monocytic leukaemia cutis.

Group 6 : Miscellaneous.

Two cases have been recorded of a generalized bronzed pigmentation, and three in which bullae appeared on the hands and feet.

Tables II–V present an analysis of some of the factors influencing the clinical course of these lesions.

TABLE II.—*Relationship of Sex to Type of Cutaneous Lesion.*

Type of rash.	Males.	Females.
Purpuric	22 (69%)	10 (31%)
Maculo-papular . . .	10 (67%)	5 (33%)
Nodular	7 (64%)	4 (36%)
Suppurative . . .	9 (90%)	1 (10%)
Exfoliative	5 (84%)	1 (16%)
All cases	71%	39%

Table II shows the relationship of sex to the type of lesion. 71 per cent of all cases occurred in males. This is very similar to the sex distribution for all cases of monocytic leukaemia (Whitby and Britton, 1946). There were no significant differences for the different lesions, except perhaps some increase in incidence in males for the suppurative and exfoliative types.

TABLE III.—*Relationship of Age to Type of Cutaneous Lesion.*

Type of rash.	Age.		
	0–19 yrs.	20–39 yrs.	40 + yrs.
Purpuric	6 (19%)	10 (31%)	16 (50%)
Maculo-papular . . .	2 (12%)	5 (32%)	9 (56%)
Nodular	1 (9%)	4 (36%)	6 (55%)
Suppurative	0 (0%)	1 (10%)	9 (90%)
Exfoliative	0 (0%)	2 (33%)	4 (67%)
All cases	7 (14%)	16 (32%)	27 (54%)

Table III analyses the age incidence of the patients. It can be seen that more than half of all cases are over the age of forty, in contrast to acute lymphatic and myeloid leukaemias. When the age incidence in relation to the type of skin lesion is considered no significant differences are to be found.

TABLE IV.—*Relationship of Duration of Survival to Type of Cutaneous Lesion.*

Type of rash.	0–3 mos.	4–9 mos.	10 + mos.
Purpuric	18 (57%)	10 (31%)	4 (12%)
Maculo-papular	7 (44%)	5 (31%)	4 (25%)
Nodular	6 (55%)	1 (9%)	4 (36%)
Suppurative	2 (20%)	3 (30%)	5 (50%)
Exfoliative	0 (0%)	2 (33%)	4 (67%)
All cases	26 (52%)	12 (24%)	12 (24%)

In Table IV the type of lesion is related to the duration of the disease.* The cases with purpura tend to be rather more acute than the others, and those with suppurative and exfoliative lesions decidedly more chronic.

TABLE V.—*Frequency of Different Types of Cutaneous Lesion.*

Type of rash.	Frequency.
Purpuric	32 (64%)
Maculo-papular	16 (32%)
Nodular	11 (22%)
Suppurative	10 (20%)
Exfoliative	6 (12%)
Miscellaneous	3 (6%)

Table V shows the frequency of the different types of rash. It will be noted that purpura is the commonest single lesion. Nevertheless, the maculo-papular and nodular varieties are by no means rare, and it will be seen by reference to Table I that in 56 per cent of cases non-purpuric rashes occurred.

PATHOGENESIS.

If the petechiae are sectioned, in some cases monocytes may be seen in the capillary adventitia and infiltrating the periphery of the extravasation. Usually, however, monocytes are only prominent inside the vessels. In the maculo-papular and nodular group the histology is diverse, but in all cases there is an infiltration by cells of the monocytic series, primitive and mature types varying in proportion. In paraffin sections stained with haematoxylin and eosin these cells are at times indistinguishable from others of the lympho-reticular series. Typically they are spheroidal, pyriform or polygonal in shape, with an indistinct cell outline and lightly eosinophilic cytoplasm. The nucleus is commonly eccentric and relatively large, showing varying degrees of irregularity in shape. From being spheroidal and then oval, it becomes reniform and then band-like, often producing a notched or lobulated appearance. The chromatin is granular, and often a hyperchromatic longitudinal groove, an effect of the nuclear folding, is seen. Nucleoli and mitoses are not prominent. In the earlier lesions the infiltration is seen between the connective-tissue fibres and in the adventitia of the small vessels. Later, the sebaceous and sweat glands and hair follicles are involved. Finally, there is dense invasion of the entire dermis and adjacent

* Survival time was taken from the first appearance of clinical symptoms or signs.

subcutaneous tissue. Zones of hyaline necrosis and of haemorrhage may appear, while the epidermis undergoes degenerative changes which may proceed to ulceration, the vessels being packed with mononuclear cells. In the suppurative conditions a central area of necrosis is surrounded by a dense zone of mononuclear infiltration. At the periphery a light infiltration mainly in the perifollicular areas is seen. The *Staphylococcus aureus* can usually be isolated from the cellular debris.

The sequence of events during the evolution of these rashes is probably as follows:—Migration of monocytes through the capillary wall occurs, or capillary haemorrhage may take place with the formation of a small local extravasation of blood cells. The monocytes then proliferate in the perivascular region and extend to the dermal connective tissue, especially the sheaths of the hair follicles and sweat glands. However, it is possible that in some cases the monocytes develop in the first place from the histiocytes found in the blood vessel, sweat gland, and hair follicle adventitiae. Many of the small vessels become occluded by monocytic thrombi, the stroma undergoing an avascular hyaline change, and later necrosis and ulceration. It is probable that the suppurative conditions develop as a result of the impairment of local defence mechanisms by monocytic infiltration. Staphylococci and other organisms lying dormant in the hair follicles are thus able to multiply freely, with resulting pustulation.

SUMMARY.

A case of monocytic leukaemia is described in which other leukaemic symptoms were preceded for several months by a recurrent furunculosis. The patient eventually developed ulcero-nodular lesions on one arm, which were proved by biopsy to be due to infiltration with monocytic leukaemia.

The clinical and pathological features of the various rashes encountered in monocytic leukaemia are discussed and their evolution postulated.

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MAMMARY TUMOURS IN HYBRID MICE: A SEX-FACTOR IN TRANSPLANTATION.

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For many experiments hybrid mice obtained by crossing two inbred strains are preferable to either of the contributory pure lines. The F_1 hybrids, although not homozygous, are uniform in genetic constitution, they manifest "hybrid vigour," and reciprocal crosses permit the study of different maternal factors in conjunction with the same genotype. Mammary tumours develop unexpectedly and erratically in some hybrid and pure line mice supposedly deficient in the maternal milk agent which, according to Bittner (1942) and others, co-operates with oestrogenic stimulation and appropriate genetic constitution to evoke mammary tumours in mice of "high cancer" strains. The mammary tumours described in this paper developed in hybrid mice which were presumed deficient in the milk agent. Several of the tumours were readily transplantable into female but not into male mice of appropriate genetic constitution, and experiments showed that hormones controlled the outcome of transplantation.

MATERIAL AND METHODS.

A breeding colony consisting of twelve C57 Black females and three R3 males was established in September, 1945. The C57 Black strain has been maintained in this laboratory by brother-sister matings since 1941 ; spontaneous mammary tumours have not been recorded in the pedigreed stock. The R3 strain has been similarly maintained since 1934, and Dmochowski and Gye (1943) recorded mammary tumours in 83 per cent of breeding females at an average age of 8·5 months. The presumed deficiency of milk agent in the C57 mothers gave reason to expect a low incidence of mammary tumours in the F_1 hybrids of the cross C57 Black females × R3 males (designated, shortly, CR). Mammary tumours developed, however, and their incidence was studied in F_1 hybrids born between January and July, 1946. During this period, litters were recorded and the mice, with few exceptions, were sexed and given identification marks at the time of weaning. Some females were kept as virgins but most were subjected to forced breeding, being continuously with males and deprived of their litters within 48 hours of birth. Some of the mice were used in fruitless experiments, particularly for intraperitoneal injections of small doses of methylcholanthrene or for transplantation experiments. The remainder suffered no experimental interference.

Tumours were registered serially as CR1, CR2, etc. Many were removed surgically. Material so obtained as well as post-mortem material was transplanted into F_1 hybrids similar to those having the primary tumours, but mostly from a different colony. Fragments of tumour were implanted subcutaneously in the flank by means of a transplanting needle. In one experiment minced tissue was injected, using a Bashford transplanting syringe. The mice used in routine transplantations were usually siblings, but for some experiments two or more litters were combined to provide requisite numbers of each sex. The growth of tumours was recorded regularly on silhouette charts.

RESULTS.

I. Occurrence of tumours.

Nine out of 71 breeding females which survived more than 6 months developed mammary tumours, and 48 are alive without tumours. One virgin female out of 22 which survived a similar period developed a tumour, and 21 are alive without tumours. The ages of the surviving mice range from about 14 to 20 months. No new tumours have been observed during the past 6 months. Particulars of the tumours are summarized in Table I, where the tumour-bearing mice are arranged in order of birth.

The distribution of the tumours suggests a strong familial factor in their incidence. Each of the four female mice of Litter 46/3 developed a tumour, as did each of the two in Litter 46/28. Two litters, 46/33 and 46/34 were mixed before the mice were marked ; consequently the mice bearing tumours CR4 and CR10 were possibly, but not certainly, sisters. Tumour CR6 developed in the only female of Litter 46/35 which survived more than 6 months.

Table I shows the age of the mice when tumours were first observed. The average tumour age was 35·8 weeks for the whole group and 33·7 weeks for the breeding females, the latter figure being almost identical with that recorded by

TABLE I.

Serial No.	Litter No.	Tumour-age (weeks).	Transplantation. ♀	♂	
CR1	46/3	39	+	+	..
CR2	46/3	39	+	+	..
CR3	46/3	44	+	+	One I.P. injection of methylcholanthrene.
CR5	46/3	46	+	+	One I.P. injection of methylcholanthrene.
CR9	46/6	55	....	....	Virgin. One I.P. injection of methylcholanthrene.
CR7	46/28	34	+	+	..
CR8	46/28	28	....	....	..
CR4	46/33–34	15	+	+	..
CR10	46/33–34	26	....	....	..
CR6	46/35	24	+	+	Biopsy.
CR6B	46/35	39	+	+	Post-mortem.

Dmochowski and Gye for R3 breeding females. The appearance of CR4 at the early age of 15 weeks is noteworthy.

Tumours developed in three mice (CR3, CR5 and CR9) which had received each one intraperitoneal injection of 0·25 c.c. of a 0·5 per cent solution of methylcholanthrene in sesame oil. It is improbable that the carcinogen induced or accelerated tumours CR3 and CR5, for tumours CR1 and CR2 developed, without carcinogen, in sibling mice at a somewhat earlier age. The effect of the carcinogen on the growth of tumour CR9 in a virgin is conjectural. Tumour CR10 developed in a mouse bearing a slow-growing implant of tumour CR1. The remaining 6 tumour-bearing mice had not been subjected to experiment.

II. Characteristics of the tumours.

The tumours were not grossly or microscopically different from the familiar mammary tumours of "high-cancer" strains or of mice of mixed ancestry as described by Cloudman (1941) and Dunn (1945). Eight tumours were removed surgically from 4 mice and 6 recurred locally. Before death 7 of the 10 tumour-bearing mice had multiple tumours. Two tumours (CR4 and CR8) regressed under observation, but soon recurred. Post-mortem examination disclosed extensive intraperitoneal dissemination of CR1. Metastatic deposits were not found. The histological structure of the tumours was unremarkable; in particular, squamous differentiation was found, in a slight degree, in only one of the many sections examined.

Transplanted tumours were likewise unremarkable in growth and structure. Secondary tumours were found in the lungs of mice bearing tumours CR1, CR3 and CR6. Some tumours exuded milky fluid on section and microscopical examination revealed pronounced secretory activity; otherwise the microscopical appearances were not distinctive.

III. Results of transplantation.

Eight tumours, from 7 mice, were transplanted into F_1 hybrids. With rare exceptions all the implants in female mice grew, but growth of 5 of the tumours was inhibited or greatly retarded in male mice as indicated by the + sign in Table I. Tumours CR1 and CR6 were examined most thoroughly, and together they illustrate the varieties of behaviour of CR tumours.

The tumour CR1 was removed surgically from a mouse which subsequently developed a recurrent tumour at the operation site and two new mammary tumours elsewhere and when killed, 10 weeks after the biopsy, had extensive growth of tumour in the abdominal cavity. The chart of the first transplanted generation (Fig. 1) shows the pronounced delay of growth in males in comparison with females. The tumour in one male was first detected 23 weeks after implan-

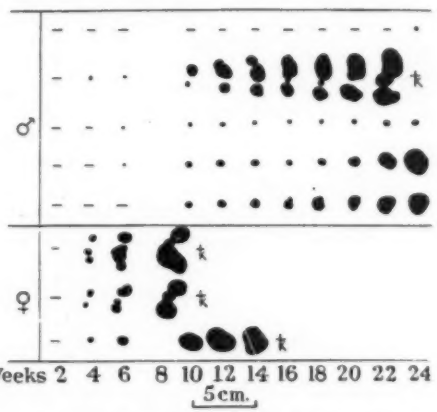

FIG. 1.—Tumour CR1. First transplanted generation.

tation, and the mouse survived a further 22 weeks before being killed with a large tumour. The difference between males and females was even more pronounced in subsequent passages (Fig. 2 and 4). In mice observed for 12 weeks after implantation, tumours grew in one out of 16 males and in all of 18 females. Many tumour-free males were killed after about 3 months, but amongst the few which were kept longer under observation tumours appeared in three at 15, 18, and 22 weeks respectively after implantation. One male survives without tumour after 26 weeks, and two after 21 weeks. The earliest tumour in a male was observed after 9 weeks, whereas all females had detectable tumours within 6 weeks. The disparity between males and females was consistent in every series of implants and persists after 5 passages. It is probable that given sufficient time implants of CR1 implants may grow almost as frequently in males as in females; the clear-cut difference is the pronounced and often extreme retardation of growth in males.

Tumour CR6 was removed surgically and implanted in 3 male and 3 female siblings. Tumours grew in all the females but in none of the males which were killed after 11 weeks. Two of the tumours in females were further transplanted

and originated two sublines differing in behaviour. The first tumour grew in each of 4 females, but not in one male, and in three subsequent passages tumours developed in 7 out of 8 females and, tardily, in 1 out of 7 males after observation for from 18 weeks in the earliest of the passages to 4 weeks in the most recent. The second tumour grew in all of 4 males and 4 females and behaved similarly in three subsequent passages, with a possible but inconspicuous delay in some of the males.

The mouse from which CR6 was removed lived a further 18 weeks and was then killed bearing two new tumours. The larger tumour, designated CR6B, was transplanted, and in three passages grew in all males as well as females with inconspicuous differences in rate. This tumour and, to a less degree, the second subline of CR6 grew more vigorously than did the more discriminating subline of CR6.

Five other CR tumours were examined in less detail. Four tumours were transplanted through only two passages and the limited observations showed that two (CR2 and CR7) resembled CR1, growing in females, and tardily or not at all in males, and two (CR3 and CR5) resembled CR6B growing with comparable vigour in males and females. Transplantation of the remaining tumour CR4 is being maintained, and up to the third passage it has resembled CR1.

A sex factor in transplantation was thus observed with five CR tumours but was inapparent with three others. It was investigated in experiments mainly with the CR1 tumour, which behaved most consistently. The possible intervention of a genetic factor or of the milk agent was investigated by implantations into reciprocal hybrids, and the more probable influence of hormones by implants in castrated males, oestrogenized males, and ovariectomized females.

Implants in reciprocal hybrids.

The tumours developed in F_1 hybrids of the cross C57 Black females $\times$ R3 males (CR) and similar CR hybrids were used for the routine transplantations. The females of the reciprocal cross, R3 females $\times$ C57 Black males (RC), have the same genetic constitution as CR females, but the males differ in their Y chromosomes, which derive from the R3 parent in CR males and from the C57 Black parent in RC males. The RC mice obtain milk containing the milk agent from their R3 mothers, whereas a supply of the agent from C57 Black mothers to the CR hybrids is uncertain.

Implants of CR1 behaved in three groups of RC siblings as in the routine transplantations. In the first group tumours grew in each of 3 females, but in none of 4 male siblings during observation for 13 weeks, in the second group in each of 2 females, but not in one male sibling during 11 weeks, and in the third group in each of 2 females, but none of 3 male siblings during 11 weeks.

Implants in castrated male mice.

Pairs of CR male litter-mates were selected from two litters of CR mice; one of each pair was castrated, the other being left as an intact control. Two weeks after operation CR1 tumour was implanted in castrated and intact males as well as in normal female litter-mates. Tumours developed, characteristically, in each of 3 females, and after long delay in one of 3 intact males, but in

none of the three castrated males, during observation for 20 weeks (Fig. 2). In an experiment to which further reference is made in the section which follows, 5 intact males and 5 castrated males were free from tumour 11 weeks after implantation of CR1. Implants of a subline of tumour CR6 grew in each of 4 intact males and in 2 of 4 castrated males. Castration, therefore, did not enhance the growth of CR tumours in male mice, but possibly increased the usual inhibition.

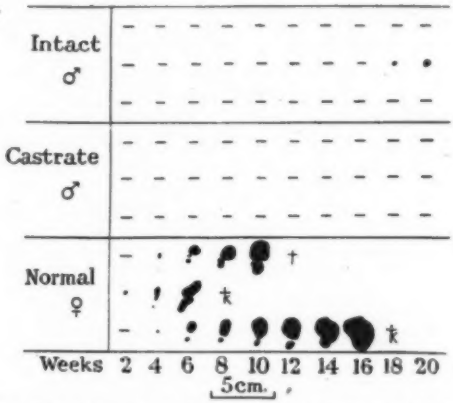

FIG. 2.—Tumour CR1. Third transplanted generation. Castration 14 days before implantation of tumour.

Implants in oestrogenized males.

Cholesterol pellets containing 25 per cent diethylstilboestrol (B.D.H.) and weighing 5–7 mg. were implanted subcutaneously in male CR mice, and normal male and female litter mates were set aside as controls. Tumour CR1 was implanted 6 days after the pellets and grew promptly in the oestrogenized males, the rate of growth being comparable with that in normal females (Fig. 3). Implants of CR1 grew similarly in 11 out of 12 males, aged about 14 months, in which stilboestrol pellets were implanted 15 days previously but not in 3 normal males of similar age. In another experiment with mice aged about 14 months, tumour CR1 was implanted in 10 intact males and 10 castrated males; 5 of the intact and 5 of the castrated mice carried stilboestrol pellets inserted 15 days previously. All the oestrogenized mice developed tumours in 5–6 weeks, whereas all the mice without oestrogen pellets were free from tumours after 11 weeks, up to which time there was no appreciable difference between intact and castrated mice.

Tumour CR4 was used in one experiment and behaved like CR1, growing in oestrogenized males and normal females, but not in normal males of the same litter.

Implants in ovariectomized females.

Fig. 4 shows the result of implanting CR1 tumour in 6 litter mates comprising 2 intact females, 2 females ovariectomized 20 days previously, and

2 intact males. Inhibition of growth in the ovariectomized females is apparent, though less complete than in intact males. In a similar experiment with RC mice, 2 ovariectomized females and 1 male were free from tumour after 11 weeks, whereas 2 intact females developed tumours within 4 weeks.

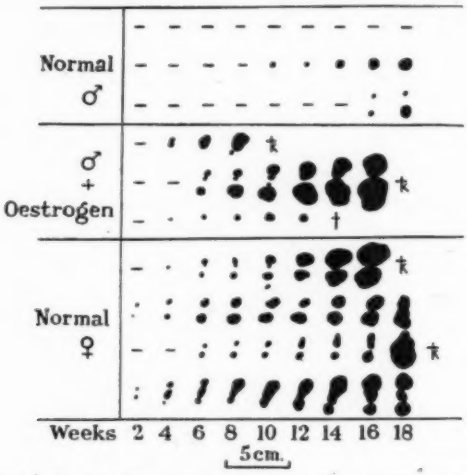

FIG. 3.—Tumour CR1. Fourth transplanted generation. Stilboestrol pellets implanted 6 days before implantation of tumour.

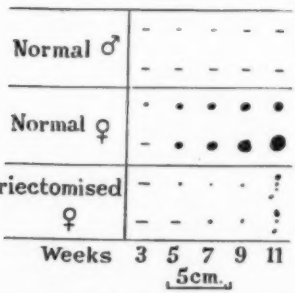

FIG. 4.—Tumour CR1. Fifth transplanted generation. Ovariectomy 20 days before implantation of tumour.

Minced CR1 tumour in a dose of 0·02 c.c. was injected subcutaneously into a litter comprising 2 ovariectomized females, 2 intact females, and 3 males. Tumours developed in ovariectomized as well as in intact females and also, within 5 weeks, in 2 of the males. Apparently the large inoculum was less dependent on hormones than the fragments implanted in the other experiments.

DISCUSSION.

The experiments show that hormones regulate the growth of implants of certain CR tumours in hybrid mice which are similar in genetic constitution to those in which the tumours originate. Genetic relationships usually predominate amongst the factors which determine the outcome of tumour transplantation, but, as Loeb (1945) maintains, they are not the only operative factors and they are not always decisive. The resistance to transplantable tumours, as studied intensively in mice of mixed ancestry, depends on genetic differences between implant and host; it is ineffective against tumours growing in their original hosts or transplanted into other animals of similar genetic constitution, and it does not account for the inhibition of CR tumours in male CR mice.

Many CR implants grow in male mice after long dormancy; possibly all will do so, given sufficient time. The inhibition in these males is not accountable to inhibition of the "take" with consequent destruction of the implant, but to a prolongation of the latent period which precedes manifest growth. Castration does not promote the growth of implants in males, whereas administration of stilboestrol enables tumours to grow as well in males as in females. Ovariectomy, in the experiments so far completed, is less decisive, but is plainly unfavourable to the growth of implants. Together, the experiments disclose the absence from normal males, and the presence in normal females, of a stimulus which is necessary for the prompt growth of CR implants. Administration of stilboestrol provides an adequate stimulus for growth in males.

The sex difference in the transplantation of CR1 tumours has been consistent through five generations of transplantation. Observations on the CR6 tumours, however, show that the dependence on the hormone stimulus is sometimes lost after transplantation, and though conspicuous in one primary tumour, it may be inapparent in another tumour developing later in the same animal. The permanence and, probably, the degree of hormone-dependence differ from one tumour to another, even when the tumours originate in the same animal. It is conceivable that many or all mammary tumours of mice pass through a phase of hormone-responsiveness which is transitory and rarely detected, but Woglom (1913), reviewing the earlier exhaustive work on transplantation of mammary tumours in mice of mixed ancestry, states that "No observer has yet discovered any difference in susceptibility between males and females . . .", and the same conclusion seems applicable to the more recent experiments using inbred mice. The CR tumours are exceptional in the existence or protraction of a hormone-sensitive phase. This exceptional property possibly, but not certainly, depends on the operation of unusual aetiological factors. Andervont (1945) provides several possible explanations for the sporadic occurrence of mammary tumours in "low-cancer" lines and hybrids presumed deficient in the milk agent; experimental evidence for one or the other is lacking. The strong familial factor in the incidence of CR tumours is under investigation. Meanwhile it is noteworthy that the CR tumours do not correspond with those mammary tumours which develop in mice deficient in the milk agent and which, according to Gardner (1947), appear at an advanced age, grow slowly, and are often of squamous cell type. The distinctive properties of CR tumours are revealed only by transplantation.

A comparable dependence on hormones is manifested by transplantable fibroadenoma of the breast in rats (*e.g.* Heiman and Krehbiel, 1936) and, in mice, by certain tumours of testis, ovary and hypophysis induced, mostly, by prolonged administration of oestrogen (Gardner, 1947). The CR tumours have several special features: they develop naturally; their aetiology is possibly unusual; they resemble the most intensively studied of animal tumours—the mammary tumours of mice of mixed ancestry and of inbred strains—except for the sex-difference in transplantation; they show that responsiveness to hormones is not inconsistent with "malignancy" as judged by the usual clinical and pathological criteria, and that responsiveness may be a temporary property characterizing one stage in the progression of a malignant tumour.

SUMMARY.

Mammary tumours were observed in hybrid mice obtained by crossing "low-cancer" strain C57 Black females and "high-cancer" strain R3 males; a low incidence of mammary tumours was anticipated on account of the presumed deficiency of milk agent in C57 Black females. A familial factor in the origin of the tumours was apparent and is under further investigation. The tumours, in structure and behaviour, resembled the familiar mammary tumours of mice, except that the growth of transplants of some of them was inhibited in male hosts. The inhibition, due to prolongation of the latent period preceding manifest growth, was annulled by administration of an oestrogen, but not by castration. Ovariectomy was unfavourable to growth in females. It was concluded that the growth of implants depended on a hormonal stimulus provided by normal females but not by normal males. A dependence on hormones was sometimes maintained through several transplanted generations, was sometimes lost in the course of transplantation, and sometimes inapparent at the first examination of a primary tumour.

It is possible that dependence on hormones characterizes one phase in the progression of malignant mammary tumours in mice, this phase, usually transitory and difficult to detect, being exceptionally prolonged in the tumours here described. As yet there is no evidence to show whether or not the exceptional property is due to unusual aetiological factors.

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SERIAL TRANSMISSION OF INFECTIOUS PAPILLOMATOSIS IN THE DOMESTIC RABBIT.

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IN a previous communication a description was given of the lesions that occurred in rabbits when the same area of skin was inoculated by scarification with a suspension of glycerolated Shope papillomas from a cottontail rabbit and a suspension of sheep dermatitis lesions (Selbie, 1946). It was observed that the two infections had a suppressing or interfering effect on each other, and that the magnitude of this effect depended on the timing of the inoculation of the two infective suspensions. The most important results of these experiments, however, were obtained from infectivity tests on the skin of normal rabbits with extracts of papillomas arising from the mixed infection. Papillomas that had arisen from skin inoculated with the two infective suspensions at different times failed to infect rabbits, and in this respect behaved in a manner to be expected of Shope papillomas in the domestic rabbit which are rarely infective. On the other hand, papillomas derived from simultaneous inoculation of both infections proved infective, and the yield of papillomas was greater than that obtained in our hands on the rare occasions that domestic rabbit papillomas had been transmitted.

In the present communication it will be shown that the papillomatous infection has been transmitted in domestic rabbits to the 10th serial passage, and that the behaviour of this infection is similar to that found in domestic rabbits that have been inoculated with virus derived from papillomas in cottontail rabbits.

MATERIAL AND METHODS.

The methods used were essentially the same as those used previously (Selbie, 1946). Market rabbits from various dealers were prepared for inoculation or treatment with croton oil by plucking the fur from one or two areas of skin on both flanks, each area measuring 6 by 8 cm. Passage of the papillomatous infection was carried out by rubbing 4 drops of a 10 per cent saline extract of papillomas from the previous passage into each of the epilated areas of skin after scarification with a needle. Papillomas used for passage, 1 to 20 in number, were removed by operation at the times indicated in Table I, except in the case of Rabbit 2, Passage 8, which was sacrificed. Extracts were prepared and inoculated immediately after removal of the papillomas except for Passage 8, in which case the extract was prepared from papillomas that had been stored at refrigerator temperature for 89 days in 50 per cent glycerol.

Two methods were used to alter the receptivity of the skin to the papillomatous infection. The first procedure was to treat the skin before inoculation with six applications of 0·3 per cent of croton oil in acetone on alternate days. In the second procedure the skin was inoculated with a mixed virus suspension

containing a 10 per cent extract of papillomas and a 10 per cent extract of another virus infection. The second virus extract was prepared from glycerolated sheep dermatitis lesions from the rabbit except in Passage 9 for Rabbits 1 and 2 and in Passage 10, where dried strawberry foot-rot lesions from the rabbit were used.

The strawberry foot-rot virus used in those experiments was first transmitted to rabbits in January, 1947, by scarification on the skin with an extract of dried lesions, supplied by Dr. J. W. Howie, of the Rowett Institute, Aberdeenshire, from an outbreak in sheep. The signs of infection are erythema at two days, haemorrhagic macules on the 3rd or 4th day, followed by crusting which, in a discrete infection, produces characteristic tall honey-coloured nodules, which usually persist up to the 12th day (Fig. 1). Microscopical examination shows that this virus infection takes an intermediate place between vaccinia and sheep dermatitis, in that vesiculation of the epidermis is followed by considerable hyperplasia of the epithelial structures (Fig. 2).

It will be seen in Table I that croton oil applications and mixed infective extracts were used in all passages from the 3rd and 4th respectively. Croton oil was applied on the right flank to one area of skin in Passage 4 and to two separate areas in Passage 3 and Passages 5 to 10. When papilloma extract alone and a mixed infective extract were used in the same rabbit, as in Passages 5 to 10, the former was inoculated in the anterior areas of prepared skin in both flanks, whereas the latter was inoculated in the two posterior areas. Thus the rabbits in Passages 5 to 10 were inoculated in four areas of skin as shown in Table I, where Areas 1 and 2 represent the anterior and posterior areas of the left flank and Areas 3 and 4 represent the corresponding areas of the right flank.

The rabbits were examined two or three times weekly from the day of inoculation to the 50th day and then at weekly intervals. The site, size and shape of papillomas were charted regularly, and caliper measurements of the papillomas were made at frequent intervals.

DESCRIPTION OF PLATES.

FIG. 1.—Discrete and confluent lesions of strawberry foot-rot infection on the flank of a rabbit 6 days after inoculation. Natural size.

FIG. 2.—Section of a strawberry foot-rot lesion in a rabbit 4 days after inoculation, showing infiltration by inflammatory cells, congestion, haemorrhage and commencing proliferation of hair follicles. H. and E. × 55.

FIG. 3.—Rabbit 1, Passage 9, Area 2, 58 days after inoculation with an extract of papillomas from Rabbit 2, Passage 8, showing typical lesions of transmissible papillomatosis. Natural size.

FIG. 4.—Rabbit 4, Passage 9, Area 2, 160 days after inoculation, showing several onion-shaped papillomas with dry horny surfaces and fleshy bases. Natural size.

FIG. 5.—Rabbit 2, Passage 2, 174 days after inoculation, showing flat disc-like papillomas with rough, dry surfaces. × 2/3.

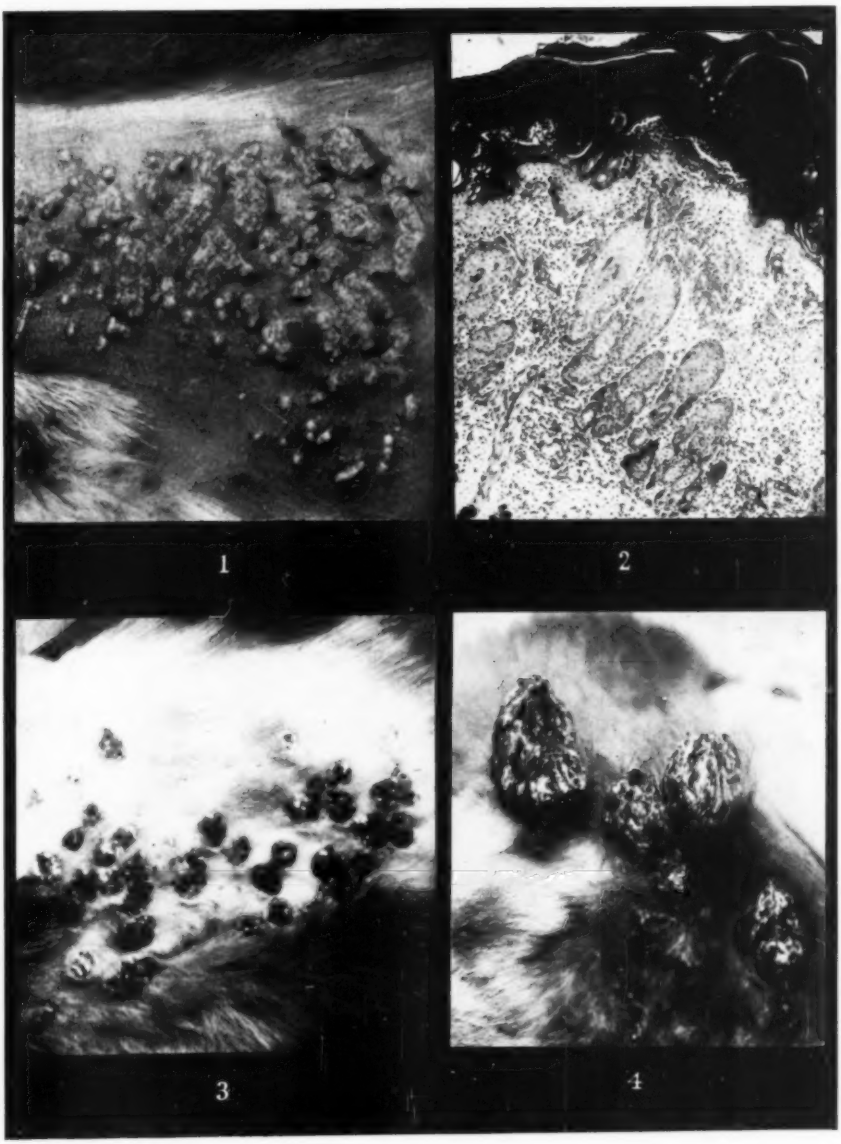
FIG. 6.—Rabbit 4, Passage, 7, Area 1, 210 days after inoculation, showing the peak-like appearances of papillomas which have united during growth. Natural size.

FIG. 7.—Rabbit 2, Passage 5, Area 1, 488 days after inoculation, showing confluent papillomatosis. The surfaces of the papillomas are dry and horny, while the bases are fleshy, and bleed readily. There is a tendency for the tumour mass to split, forming ridges. Natural size.

FIG. 8.—Rabbit 3, Passage 5, Area 2, 429 days after inoculation, showing curved, dry, keratin horns arising from fleshy bases. An ulcerating carcinoma is also shown at the base of one of the horns. Natural size.

FIG. 9.—Section from Area 2, Rabbit 4, Passage 9, 86 days after inoculation, showing a pedunculated papilloma with abundant keratin formation. H. & E. × 7.

FIG. 10.—Section from Area 3, Rabbit 1, Passage 3, 51 days after inoculation, showing a sessile papilloma with moderate keratin formation. H. & E. × 7.



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RESULTS.

Serial Passage of Papillomatosis.

The experiments which provided the first and second passages of the serial transmission of virus papillomatosis in domestic rabbits have been described in the earlier communication (Selbie, 1946). In the first experiment four rabbits were inoculated by scarification in three areas of the skin of the flanks with a mixture of an extract of glycerolated Shope papillomas from a cottontail rabbit and an extract of fresh sheep dermatitis lesions from a rabbit. The papillomas that arose from the mixed infection were at first somewhat modified by the accompanying dermatitis lesions and also by pre-treatment of the skin with croton oil and a carcinogenic hydrocarbon in two of the inoculated areas, but in their later development they differed little from typical Shope papillomas. After 10 months the only surviving rabbit was killed and a saline extract of papillomas from this rabbit was inoculated in the skin of two rabbits, which are here referred to as Rabbits 1 and 2 of the second passage (Table I).

The results of Passages 2 to 10 are summarized in Table I, where the yield of papillomas obtained for each rabbit in the direct passage line is given according to the treatment applied to the inoculated skin or to the extract used for inoculation. It will be seen that all but 1 of the 27 rabbits used were successfully infected. There was, however, considerable variability in the yield of papillomas obtained in successive passages, and in this respect the experiments reflected the usual difficulties that are met with in establishing a virus infection in a new host. Variations in the susceptibility of the rabbits probably played a considerable part in producing this effect, as is shown by the poor yields obtained in Rabbit 3, Passage 5, and Rabbit 3, Passage 9, compared with the large crops of papillomas in the other rabbits of these two passages. Variations in the infectivity of the papillomas would also appear to account for the contrast between the high yields of papillomas in Passages 3, 5 and 9 and the low yields in other passages. Thus the low yields obtained in Passages 7 and 8 could be attributed to lowered infectivity of the passage material due to prolonged storage of the papillomas used for Passage 7 and the age of papillomas used for Passage 8.

In these experiments, however, the procedures that were used to alter the receptivity of the rabbit skin apparently helped to maintain the success of serial passage. Higher yields of papillomas were obtained on skin pre-treated with croton oil than on normal skin in 6 out of 25 rabbits in Passages 3 to 10. The admixture of a second infective suspension at the time of inoculation also led to a greater yield of papillomas than the papilloma extract alone in 6 out of the 20 rabbits in Passages 5 to 10, and would also appear to account for the greater success of inoculation in Rabbits 3 and 4 as compared with Rabbits 1 and 2 in Passage 4.

Of much greater importance, however, was the effect that these procedures had in producing the sudden increases in the yields of papillomas from Passage 4 to 5 and from Passage 8 to 9. These increases in yield were undoubtedly due to the high infectivity of the extracts used for inoculation, and these were prepared from papillomas that had arisen from the inoculation of mixed virus suspensions on skin treated with croton oil. An experiment which was designed to demonstrate this effect is shown in Table II. Here it can be seen that extracts

of papillomas arising from skin treated with croton oil, or from the inoculation of a mixed infective suspension, gave a higher yield of papillomas than extracts of papillomas arising from the inoculation of papilloma extract alone on normal skin, and that the highest yield was obtained from papillomas arising from inoculations in which both measures had been used.

TABLE I.—*Passage of Virus Papillomatosis in Domestic Rabbits.*

Treatment of inoculated skin and papilloma extract :—
Skin area 1 : No treatment of skin or extract.
" " 2 : Papilloma extract mixed with extract of sheep dermatitis lesions or (only in Rabbits 1 and 2, Passage 9 and in Passage 10) strawberry foot rot lesions.
" " 3 : Pre-treatment of skin with croton oil.
" " 4 : Treatments 2 and 3 combined.

Passage number.	Age of papilloma used for passage (days).	Rabbit number.	Yield of papillomas on skin area :— 1.	2.	3.	4.	Regressive changes.
2	292	1	10	..	..	..	Complete
		2	10*	..	..	..	Temporary
3	77	1	55	..	200+*	..	0
4	51	1	1	..	2	..	0
		2	0	..	0	..	0
		3	..	0	..	6	0
		4	..	0	..	20*	0
5	56	1	60	40	35	60	Temporary
		2	100+	40	40	40	0
		3	0	60	9	4	0
		4	80*	100	80*	60	Complete
6	35	1	2	2	40	40	0
		2	16	14	60	60*	0
7	76	1	1	1	0	2	0
	(+ 89 days in 50% glycerol)	2	0	0	1	0	Complete
		3	0	0	1	0	..
		4	6	3	11*	1	0
8	112	1	0	3	0	1	Complete
		2	0	0	0	1*	0
9	70	1	7	50+	6	50+	0
		2	30	50+	40	50+	0
		3	7	0	4	0	Complete
		4	30*	50+*	30*	50+*	0
10	86	1	5	7	10	20	0
		2	7	0	1	3	Complete
		3	11	12	14	4	0
		4	18	7	10	15	0

* Papillomas for next passage taken from this skin area. .. = not done.

TABLE II.—*Infectivity of Papillomas from Rabbit 4, Passage 5.*

Papillomas from—	Yield of papillomas on normal skin. Rabbit A.	Rabbit B.
Area 1 (no treatment)	8	4
Area 2 (mixed infective suspension)	10	17
Area 3 (croton oil pre-treatment)	13	10
Area 4 (mixed infective suspension + croton oil pre-treatment	35	30

The successful outcome of the experiments could thus be attributed to a number of factors, of which the most important was the use of mixed virus suspensions and pre-treatment of the skin with croton oil. It will, however, be noted particularly in Passages 5 and 9 (Table I) that these measures had little or no stimulating effect on the infection when good yields were obtained without their aid. It would thus appear that the results obtained with each inoculation depended on the interplay of many factors concerning the nature of the extract used for the inoculation, the receptivity of the susceptible cells and the general susceptibility of the host.

Description of the Infection.

Incubation period.

There was considerable variation in the times at which papillomas first appeared. Where the eventual yield was good, as in Passages 5 and 9, papillomas were usually first detected between the 18th and 21st days, but in some instances, especially where the yield was poor, as in Passages 4 and 8, the first papillomas did not appear till after 35 to 49 days. There was also considerable variation in the times of appearance of papillomas in single rabbits. In most rabbits the majority of the papillomas were detected within 6 days following the appearance of the first papilloma, but papillomas continued to arise for a further 10 to 14 days. Pre-treatment of the skin with croton oil lengthened the incubation period when this procedure did not increase the yield of papillomas, as in Passage 5, where papillomas were first seen in the treated skin 5 to 10 days later than in the normal skin. On the other hand, when the yield of papillomas was increased by this procedure there was also a shortening of the incubation period as in Passage 6, where papillomas first appeared on the treated skin at 21 days and in untreated skin at 25 days.

The admixture of sheep dermatitis virus always appeared to lengthen the incubation period of the papillomas, because at the time early papillomas would be expected, that is between the 18th and 21st day, they were hidden by the dermatitis lesions, which showed little sign of abatement up to the 28th day. On the other hand, the admixture of strawberry foot-rot virus, which gives rise to a short-lived infection that is usually clear by the 18th day, did not interfere with the detection of early papillomas, and had no apparent influence on the length of the incubation period.

The development of papillomas.

An initial stage of rapid growth lasting for 2 or 3 months was followed by a period of slow general enlargement to an almost stationary condition lasting up to 9 to 12 months. Finally, in some instances growth was resumed with downward extension and thickening of the bases of the papillomas, and other manifestations of malignancy.

In general, 3 weeks after their first appearance discrete papillomas reached a diameter of 0·5 cm., which was doubled during the next 4 weeks. Thereafter the rate of growth varied considerably, discrete papillomas reaching a diameter of 2·5 cm. at 100 to 150 days, whereas in confluent papillomatosis the individual papillomas did not exceed 0·9 to 1·2 cm. in diameter. The height also varied considerably, according to the type of papilloma that arose; usually they were

found to be 1 to 2 cm. in height at 150 days, but in Rabbit 3, Passage 5, and Rabbit 1, Passage 7, horns developed, measuring up to 3·5 cm. in height (Fig. 8).

Regressive changes in the papillomas were observed in 9 of the 27 rabbits used in Passages 2 to 10 (Table I). They were first observed within a period of 40 days after the papillomas were first noted, except in Rabbit 2, Passage 2, and Rabbit 1, Passage 8, where regression was first detected at 160 and 84 days respectively. The regressive changes led to a complete disappearance of all papillomas in 7 rabbits, but in Rabbit 2, Passage 2, growth was eventually resumed in all the papillomas, while in Rabbit 1, Passage 5, all the papillomas disappeared except 14, which survived regression and ultimately resumed rapid growth. It will also be noted in Table I that complete regression was usually associated with a low yield of papillomas.

Appearance of papillomas.

Macroscopical.—In appearance the papillomas were essentially the same as those described in papillomatosis in the domestic rabbit by Shope (1933). When first observed the papillomas looked like minute red or pink seeds in the skin, frequently with a small scab on the surface. In the course of a few days the lesions were 1 to 2 mm. across, slightly raised, pearly pinkish or grey to black in colour, and assumed a typical warty appearance within 10 days. At a later stage in their development the papillomas had thick fleshy bases, while the surface was dry and rough, as is shown in Fig. 3 of an area of skin 58 days after inoculation. As the papillomas grew, different shapes and sizes arose, but while the degree of crowding obviously altered the shape of the papillomas, there was a general tendency in any one rabbit for the papillomas to be of one type. Thus the flat disc-like papilloma (Fig. 5) was the only type in Rabbit 2, Passage 2, while the majority of the papillomas in Rabbit 4, Passage 9, were pedunculated onions with dry horny tips (Fig. 4). In confluent papillomatosis there were craggy ridges of papillomatous tissue (Fig. 7), while, where a few papillomas united, a peak-like appearance was common (Fig. 6). In some cases the formation of keratin so predominated that long horns developed (Fig. 8).

Microscopical.—The microscopical appearances of the papillomas were similar to those described by Weston Hurst (Shope, 1933), who noted that there was no essential difference between the papillomas procured in cottontail rabbits and domestic rabbits inoculated with cottontail material. The architecture was that of a typical papilloma with a central stroma containing blood vessels and connective tissue derived from the dermis, supplying masses of rapidly proliferating epithelium. The layers of the epithelium in which no inclusion bodies were seen were greatly thickened, and surmounted by a mass of keratin. The general histological arrangement of cells was the same whether the papillomas were pedunculated with abundant keratin (Fig. 9) or flat discs with little keratin formation (Fig. 10).

DISCUSSION.

In his original experiments on infectious papillomatosis of the rabbit Shope (1933) found that the disease could be transmitted in series through the cottontail rabbit, the original host, but that although virus of cottontail origin was infective to the domestic rabbit, the infection could not be transmitted in series through

the domestic rabbit. In later experiments, Shope (1935) recovered active virus from a number of papillomas in domestic rabbits and established several transmissible strains, of which one was subsequently reported to have reached the 14th serial passage in the domestic rabbit (Shope, 1937). Other workers have also found that extracts of papillomas from domestic rabbits are occasionally infective, and that their infectivity can be enhanced either by pre-treatment of the inoculated skin, as with methylcholanthrene or turpentine and acetone (Friedewald, 1942), or by treatment of the papilloma extract as with trypsin (McIntosh and Selbie, 1938), but none, apart from Shope, has hitherto reported transmission beyond the second passage. The experiments described here have confirmed Shope's finding that this infection can be transmitted in series in the domestic rabbit. Furthermore, the high proportion of rabbits successfully infected and the comparatively high yields of papillomas obtained with this strain of papilloma virus indicate that there has been an adaptation of the virus to the new host. This adaptation of the virus would have occurred during the first passages in association with superinfection by sheep dermatitis virus (Selbie, 1946).

The papillomas that are produced by the transmissible infection have been shown to be similar to those produced by the inoculation of cottontail material in their manner of growth and in their naked-eye and microscopical appearances. They also share the property of regularly developing into frankly malignant growths, as will be described in a later communication. Furthermore, evidence is forthcoming from serological tests that the infecting agent in the transmissible papillomas is closely related to the virus in cottontail papillomas. This experimental infection thus provides unique material for the further elucidation of the problems that have arisen in the study of the Shope rabbit papilloma, in that the papillomas are regularly infective and also progress regularly to the manifestations of malignancy—two conditions which are but rarely obtained together either in the cottontail rabbit or in the domestic rabbit infected with cottontail material.

Although this transmissible papillomatosis is now in its 12th serial passage, the latent period is somewhat longer and the yields of papillomas are generally smaller than those that usually follow inoculation with extracts of papillomas from the cottontail rabbit. The application of croton oil to the skin before inoculation and the admixture of a second virus suspension with the inoculum have in some cases increased the crop of papillomas, and have apparently been responsible for the increased infectivity of the papillomas used for Passages 5 and 9. There is, however, no evidence of a general increase in the infectivity of papillomas, since the later passages are giving yields of papillomas that are no greater than those in the earlier passages. It would therefore appear that there has been no further adaptation of the virus in spite of repeated application of the measures that were used when adaptation occurred in the first passage.

The generally low infectivity of the transmissible papillomas and the apparent lack of further adaptation during passage may, however, reflect a fundamental difference in the nature of the infection in cottontail and domestic rabbits. Shope (1935) has suggested that papillomas in the domestic rabbit contain a lower concentration of available virus than in the cottontail rabbit. Moreover, Beard, Bryan and Wyckoff (1939) have isolated by centrifugalization a heavy protein from cottontail papillomas in quantities proportional to infectivity and varying from 0·008 mg. to 1·0 mg. per g., but they have obtained no such protein from

papillomas in the domestic rabbit. Bryan and Beard (1949) have also calculated that the number of units of this heavy protein corresponding to a 50 per cent end-point infectious unit is about 94 million. As Friedewald (1942) points out, this figure probably expresses the difficulty of bringing virus in association with susceptible cells, but its magnitude along with the failure to detect virus protein in domestic rabbit papillomas would suggest that the low infectivity of papillomas in the domestic rabbit is due to a low concentration of virus in the papilloma cells. This view has also been supported by serological observations, which have shown that less circulating antibody is produced by the infection in the domestic rabbit than in the cottontail (Kidd, 1938*a*), and that extracts of papillomas from the domestic rabbit contain less specific antigen than those from the cottontail (Kidd, 1938*b*). It is thus possible that the main difference in the nature of the infection in the two species of rabbit is that the lesion in the domestic rabbit is produced with much less multiplication of virus in the affected cells—a phenomenon well exemplified by the bacteriophages, which can produce lysis of bacteria with burst sizes ranging from a very few to 200, representing the amount of multiplication that has taken place within the bacterium (Delbrück, 1942). If this is so it follows that the papilloma virus, once it has gained entry to the susceptible cells, is more virulent to the domestic rabbit, and this would also account for the lesions being of a more progressive nature than in the cottontail rabbit.

Although it would appear likely that there is less multiplication of the papilloma virus in the domestic rabbit, there are undoubtedly other factors that contribute to the difficulty of transmitting these papillomas. Shope (1933) has shown that extracts of domestic rabbit papillomas regularly contain an inhibiting substance which can greatly reduce the infectivity of extracts of cottontail papillomas. It has been suggested that this inhibiting substance may be extravasated antibody, but in the cottontail rabbit this inhibitory effect is observed only in large, disorderly, fissured and inflamed papillomas and when antibody is present in the blood in high titre (Kidd, 1939). Thus, although antibody may sometimes take part in masking the virus, it would appear that some other factor is responsible. It may be that the host-parasite relationship is such that the virus is difficult to extract from the infected cells, or that much of the virus is inactive and proves inhibitory by interfering with the access of active virus to the susceptible cells. In any case it is evident that much work has yet to be done on the questions arising from the apparent paradox of the relative non-infectivity and progressive nature of papillomatosis in the domestic rabbit, and there is no doubt that the transmissible strain described here will provide useful material for the further investigations of these problems.

SUMMARY.

Shope's finding that infectious papillomatosis of the cottontail rabbit can be transmitted in series in the domestic rabbit has been confirmed.

It has been shown that the transmissible infection behaves in a manner similar to the infection produced by the inoculation of extracts of cottontail papillomas in the domestic rabbit.

The results of the first ten serial passages are described, and the part played

by ancillary measures in maintaining the transmissibility of the infection is discussed.

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THE ROLE OF CROTON OIL APPLICATIONS, ASSOCIATED WITH A SINGLE PAINTING OF A CARCINOGEN, IN TUMOUR INDUCTION OF THE MOUSE'S SKIN.

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IT was previously shown, in following up the mechanism of the co-carcinogenic action of croton oil (Berenblum, 1941 *a* and *b*), that the latent period of tumour induction, following an adequate period of painting of the mouse's skin with a carcinogen, was not influenced when *preceded* by 26 weeks of croton oil applications. Yet, a significant increase in tumour incidence was observed when the croton oil treatment was given *subsequent to* a sub-optimal period (8 weeks) of painting with a carcinogen. It was concluded that two independent processes were involved in the evolution of a visible wart : a *precarcinogenic action*, elicited by carcinogens but not by croton oil, and therefore of a specific nature ; and an *epicarcinogenic* action, representing the formation of a visible tumour (wart) at a site previously " prepared " by sub-optimal treatment with a carcinogen, and elicited by a non-carcinogenic agent, such as croton oil, as readily as by continued application of the carcinogen itself, and therefore non-specific (Berenblum, 1941*b* ; 1944).

The problem was further investigated by Mottram (1944 *a* and *b*), who claimed (*a*) that the procedure could be simplified, in that a *single* application of a carcinogen (3:4-benzpyrene), followed by repeated applications of croton oil, was sufficient to lead to tumour production ; and (*b*) that this effect was accentuated when the croton oil was applied *before* as well as *after* the single

application of the carcinogen. Mottram concluded that carcinogenesis was composed of 3 phases :

(1) A " Sensitizing Factor," which could be brought about by preliminary treatment with croton oil, acting presumably in a non-specific manner by causing hyperplasia, on the supposition that proliferating cells were more responsive than resting cells to the specific carcinogenic action which followed.

(2) A " Specific Cellular Reaction," induced by the specific action (even of a single application) of a carcinogen, this representing the essential neoplastic change.

(3) A " Developing Factor," responsible for the actual appearance of a visible wart, and produced by croton oil or by a carcinogen.

Thus, Mottram's second stage (" Specific Cellular Reaction ") corresponds to the " Precarcinogenic Action " of Berenblum (1941*b* ; 1944) and also to the " Initiating Process " of Friedewald and Rous (1944), while his third stage (" Developing Factor ") corresponds to Berenblum's " Epicarcinogenic Action " and Friedewald and Rous's " Promoting Process." (See Table of corresponding nomenclatures, Berenblum, 1947.) However, Mottram's first stage (" Sensitizing Factor ") is a new concept, not brought out by any of the previous workers. Unfortunately, his results were based on experiments which, owing to the prevailing war-time conditions, were performed on inadequate numbers of animals, and therefore call for confirmation.

The present work constitutes a repetition and extension of Mottram's experiments, designed to test the two principal claims :

(*a*) whether a single application of a carcinogen is indeed adequate antecedent treatment to allow the " developing " effect of croton oil to be manifested ; and

(*b*) whether there is in fact an initial, non-specific, hyperplasia-producing, " sensitizing factor," operating prior to the specific carcinogenic action.

METHODS.

Mice of mixed strain (white and coloured) from this laboratory stock were used, and were maintained throughout the experiment on an adequate mixed diet. The experimental area of skin, in the interscapular region, was clipped periodically with fine scissors for the removal of hair, and the test solutions applied with a glass rod.

In order to diminish variability in response, all reagents were applied in the form of solutions in the non-volatile liquid paraffin, instead of in such volatile solvents as acetone or benzene. This necessitated, however, the use of higher concentrations, as was demonstrated elsewhere (Berenblum and Schoental, 1947).

EXPERIMENTAL.

In the first experiment two groups of mice were treated as follows :

Group A : 50 mice were given paintings of 5 per cent croton oil in liquid paraffin twice-weekly for two weeks, then given a single painting

of 0·8 per cent 3:4-benzpyrene in liquid paraffin, and then painted twice-weekly for 20 weeks with the croton oil solution.

Group B : 45 mice were given identical treatment to those of Group A, except for the omission of the pre-painting with croton oil.

TABLE I.

Series.	Treatment. *			Number of mice used.	Survivors at time of 1st tumour.	Number of mice with tumours.	Percentage of mice bearing tumours.	Average latent period† (weeks).
	a.	*b.*	*c.*					
A	Cr. oil	BP	Cr. oil	50	45	12	26·5	10·8
B	—	,,	,,	45	40	15	37·5	10·6
I	Cr. oil	DMBA	,,	48	42	24	57	8·1
II	—	,,	,,	45	37	20	54	7·5
III	Cr. oil	,,	—	46	(35)	0	0	—
IV	—	,,	—	45	38	1	2·6	—
V	Cr. oil	—	Cr. oil	48	(41)	0	0	—

* Column *a* : 5 per cent croton oil in liquid paraffin twice-weekly for 2 weeks.
,, *b* : BP = 0·8 per cent benzpyrene in liquid paraffin applied once only.
DMBA = 1·5 per cent 9:10-dimethyl-1:2-benzanthracene in liquid paraffin applied once only.
,, *c* : 5 per cent croton oil in liquid paraffin twice-weekly for 20 weeks.
† Latent period counted from time of commencement of application of second croton oil treatment.

From Table I it can be seen that there was little difference between the two groups, either in the total number of tumours induced or in the relative latent periods of tumour induction. The small differences observed are not significant, and, in any case, are the opposite to those reported by Mottram, since the animals pre-treated with croton oil actually developed *fewer* tumours than those without pre-treatment.

A second, and more elaborate, experiment was carried out, in which a 1·5 per cent solution in liquid paraffin of 9:10-dimethyl-1:2-benzanthracene (DMBA) was used as the carcinogen (single painting) ; and the experiment also included three additional control groups :—

Group I : 48 mice were painted twice-weekly for 2 weeks with croton oil, then given one application of DMBA, and then painted twice-weekly with croton oil for a further 20 weeks.

Group II : 45 mice were treated as those in Group I, except for the omission of pre-painting with croton oil.

Group III : 46 mice were given croton oil for 2 weeks, twice-weekly, and then 1 application of DMBA, but not followed by croton oil treatment.

Group IV : 45 mice were given a single application of the carcinogen (DMBA), and nothing else.

Group V : 48 mice received croton oil twice-weekly for 23 weeks (i.e. similar to those in Group I, except for the omission of application of DMBA).

It can be seen (Table I) that, here again, no significant difference in tumour yield between those pre-treated with croton oil (Group I) and those without

such pre-treatment (Group II). In the control groups no tumours developed except for an isolated tumour in Group IV.

DISCUSSION.

The results described above lend no support to the assumption that an induced hyperplasia *preceding* the painting of a carcinogen has any augmenting influence on experimental tumour induction. This would seem to eliminate Mottram's postulated "Sensitizing Factor."

On the other hand, Mottram's other result, that a *single* application of a carcinogen, followed by applications of the irritant, croton oil, will induce tumours, received confirmation. This latter finding accords well with both Berenblum's and Rous's concepts of a "precarcinogenic" or "initiating" phase, followed by an "epicarcinogenic" or "promoting" phase.

Mottram's simplification of procedure (involving a single application of a carcinogen) has important technical significance, enabling the various phases of carcinogenesis to be analysed in a more accurate and quantitative fashion than has, up to the present, been possible (Berenblum and Shubik, 1947.)

SUMMARY.

1. Mottram's finding that a single application of 3:4-benzpyrene, followed by repeated applications of croton oil, will induce tumours, is confirmed, and is shown to hold also for 9:10-dimethyl-1:2-benzanthracene.

2. Mottram's other observation, that non-specific hyperplasia, induced by croton oil *previous to* the single application of a carcinogen, leads to an increase in the number of tumours induced, is not confirmed.

We wish to thank Mr. H. W. Wheal for technical assistance and the care of the animals.

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A NEW, QUANTITATIVE, APPROACH TO THE STUDY OF THE STAGES OF CHEMICAL CARCINOGENESIS IN THE MOUSE'S SKIN.*

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FROM the previous examination of the role of croton oil in chemical carcinogenesis (Berenblum and Shubik, 1947), it became apparent that the use of a *single* application of a carcinogen, followed by repeated applications of croton oil (Mottram, 1944 *a* and *b*) could serve as a "model experiment" for the more accurate analysis of the component phases of carcinogenesis. In the present communication, certain propositions, which would appear to be the logical explanation of previously observed phenomena, have been put to the test by this new, quantitative approach.

When carcinogens are repeatedly applied to the skin of mice, and the resulting tumour incidences are plotted against time, the curves obtained are usually of the type represented by *a*, *b* and *c* in Fig. 1, the positions of the curves along the time axis (i.e. the latent periods) varying according to the carcinogen used, but the heights of the curves always approximating to 100 per cent of the surviving animals. However, when the carcinogen is applied for a sub-optimal period of 8 weeks (Berenblum, 1941*b*), or once only (Mottram, 1944 *a* and *b*; Berenblum and Shubik, 1947) and the skin is thereafter treated with croton oil, the tumour incidence curve, though beginning as early as, or even earlier than, those of continuous carcinogen treatment, rapidly reaches a set level well below 100 per cent, and remains at that level *however long the croton oil treatment is continued* (see curve *x*, Fig. 1).

This could be explained by considering the preliminary carcinogenic action as causing an irreversible conversion of a few normal cells into a few latent tumour cells, and by assuming that the croton oil converts these latent tumour cells into visible tumours. (A similar view has been put forward by Friedewald and Rous (1944 *a* and *b*) to explain the fact that chemically-induced rabbit skin papillomas can, after regression, be made to reappear by non-specific forms of irritation.)

On this basis, the height of curve *x* (Fig. 1) could be taken as a measure of the preliminary action of the single painting with the carcinogen, while the position of the curve along the time axis (i.e. the latent period) would represent a measure of the subsequent action by the croton oil treatment.

The correctness of this interpretation could readily be tested as follows: A change in the method of initial treatment (by using a different carcinogen, or by altering its concentration, or by varying both factors at the same time) should

* A preliminary communication of this work was presented by Dr. I. Berenblum at the Fourth International Cancer Congress at St. Louis, in September, 1947.

influence the height of the curve of the "model experiment" *without altering the latent period;* on the other hand, a change in the croton oil procedure should influence the latent period *without affecting the height of the curve.* The latter

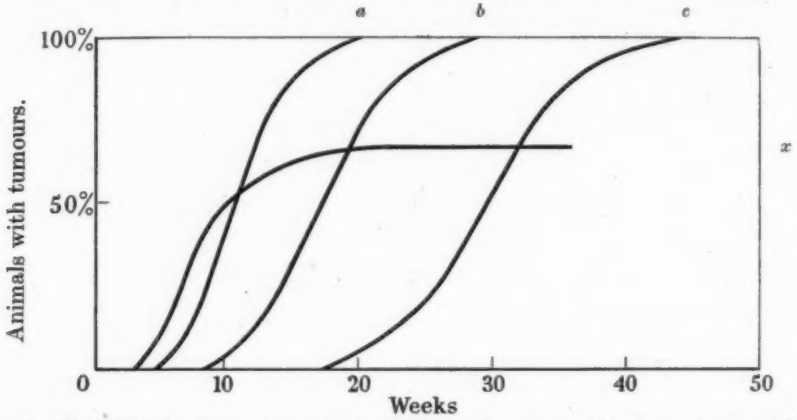

FIG. 1.—Characteristic curves for tumour-production in groups of mice: A comparison between the effect of painting skin continuously with carcinogens of high potency (curve *a*), moderate potency (curve *b*), and low potency (curve *c*), and that obtained by a single painting with a potent carcinogen followed by repeated painting with croton oil (curve *x*).

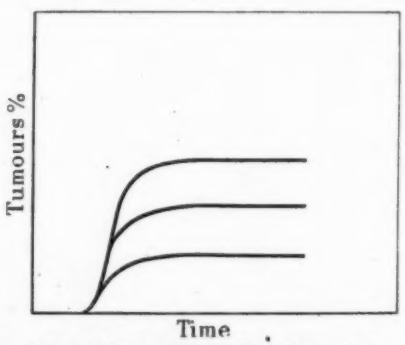

FIG. 2.—Type of curves anticipated in the 'Model Experiment' when different carcinogens are used for the single, initial painting.

test could be performed either by altering the concentration of the croton oil, or, more effectively, by delaying the croton oil treatment for a given period, and noting whether the latent period is correspondingly delayed, without diminution in tumour incidence.

For purposes of illustration, these relationships could be represented graphically, Fig. 2 showing the varying heights of the curve to be expected when different carcinogens are used, and Fig. 3 showing how the curve should be shifted along the time axis with delays in the croton oil treatment.

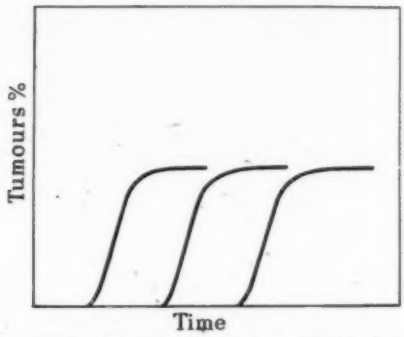

FIG. 3.—Type of curves anticipated in the 'Model Experiment' when the carcinogen is kept constant, but the croton oil treatment is delayed for different periods.

METHODS.

Mice of mixed strain (white and coloured) from this laboratory stock were used, and were maintained throughout the experiments on an adequate mixed diet. The experimental area of skin, in the inter-scapular region, was clipped periodically with fine scissors for the removal of hair, and the test solutions were applied with a glass rod. As in the previous communication (Berenblum and Shubik, 1947), liquid paraffin was, wherever possible, used as solvent, to diminish variability in response. This necessitated the use of higher concentrations, as demonstrated by Berenblum and Schoental (1947).

EXPERIMENTAL.

Comparison of different carcinogens in the "Model Experiment."

Group A (45 mice): A single application of a 0·8 per cent solution of 3:4-benzpyrene in liquid paraffin was applied to the mouse's skin, followed, after a 3-day interval, by twice-weekly applications for 20 weeks of a 5 per cent solution of croton oil in liquid paraffin. (This group is from a previous investigation—Berenblum and Shubik, 1947.)

Group B (64 mice): A single application of a 0·3 per cent solution of 1:2:5:6-dibenzanthracene in benzene, followed, after a 3-day interval, by twice-weekly applications for 20 weeks of the croton oil solution. (Benzene was used as solvent in this case, because dibenzanthracene is very sparingly soluble in liquid paraffin.)

Group C (92 mice): A single application of a 1·5 per cent solution of 9:10-dimethyl-1:2-benzanthracene in liquid paraffin, followed, after a 3-day interval, by twice-weekly applications for 20 weeks of the croton oil solution.

As shown in Table I, the tumour incidence was 39·5 per cent. in the benzpyrene series, 29·5 per cent in the dibenzanthracene series, and 58 per cent in the dimethyl-benzanthracene series; yet the latent periods for the three groups were almost identical (i.e. 10·6, 10·1 and 9·5 weeks respectively). It is interesting to note that when these three carcinogens are applied continuously (twice-weekly) throughout the experiment, the corresponding latent periods are 16, 33 and 11 weeks respectively.

TABLE I.

Series.	Treatment *	Number of mice used.	Survivors at time of 1st tumour.	Mice with tumours.	Percentage with tumours.	Average latent period (weeks).
A	BP and croton oil	45	40	15	37·5	10·6
B	DBA and croton oil	64	37	11	29·5	10·1
C	DMBA and croton oil	92	62	36	58	9·5

* Treatment consisted of one application of the carcinogen, followed by twice weekly applications of croton oil solution for 20 weeks.
Carcinogen: BP = 0·8% 3:4-benzpyrene in liquid paraffin.
DBA = 0·3% 1:2:5:6-dibenzanthracene in benzene.
DMBA = 1·5% 9:10-dimethyl-1:2-benzanthracene in liquid paraffin.

Effect of interval between the initial, single, application of a carcinogen and the subsequent croton oil treatment.

For this experiment, the most potent of the three carcinogens (9:10-dimethyl-1:2-benzanthracene), as a 1·5 per cent solution in liquid paraffin, was used for the single application, but the croton oil treatment (5 per cent in liquid paraffin, twice-weekly for 20 weeks) was instituted after different intervals, as follows :*

Group C (92 mice): an interval of 3 days only. (This group, serving a control for the series, was from the previous experiment, see above.)
Group D (92 mice): an interval of 5 weeks.
Group E (95 mice): an interval of 10 weeks.
Group F (45 mice): an interval of 15 weeks.
Group G (46 mice): an interval of 20 weeks.

The numbers of animals with tumours, developing each week, are shown in Table II, and the results summarized in Table III.

TABLE III.—*The Influence of Interval Between the Single Application of Carcinogen (DMBA) and the Croton Oil Treatment (Twice Weekly for 20 Weeks).*

Series.	Interval.	Number of mice used.	Survivors at time of 1st tumour.	Mice with tumours.	Percentage with tumours.	Average latent period (weeks). (a).	Average latent period (weeks). (b).
C	3 days	92	62	36	58	9·8	9·5
D	5 weeks	92	80	46	57·5	11·2	6·2
E	10 ,,	95	48	35	73	16·8	6·8
F	15 ,,	45	24	18	75	23·6	8·6
G	20 ,,	46	26	15	57·5	25·5	5·5

(*a*) Latent period counted from the commencement of the experiment.
(*b*) Latent period (*a*) with interval deducted.

* For controls of carcinogen alone, and croton oil alone, see preceding paper (Berenblum and Shubik, 1947).

TABLE II.

Series.	Interval between carcinogen (DMBA) and croton oil.	Number of animals used.	Survivors at time of first tumour.	Tumour production per week (animals bearing warts).*																		Total.
				2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	
Q	3 days	92	62	—	—	—	—	2	3	10	7	5	3	3	1	1	—	—	—	—	—	1	36
R	5 weeks	92	80	3	—	7	9	4	10	8	4	—	—	1	—	—	—	—	—	—	—	—	46
S	10 „	95	48	—	1	6	9	2	2	7	4	—	1	1	2	—	—	—	—	—	—	—	35
O	15 „	45	24	—	—	—	3	2	4	1	1	3	2	—	—	—	2	—	—	—	—	—	18
M	20 „	45	26	1	—	1	8	0	3	2	—	—	—	—	—	—	—	—	—	—	—	—	15

* From time of first application of croton oil (i.e. excluding intervals).

It will be seen that even after as long an interval as 20 weeks between the initial painting with the carcinogen and the subsequent croton oil treatment, the total tumour incidence (Table III) remained undiminished. The latent period was delayed, however, in each case by a period approximately corresponding to the interval free from treatment. (The detailed results, shown in Table II, indicate that early tumour development, judged in each case from the commencement of croton oil treatment, was commoner in the experimental groups D–G than in the control group C. Thus, in 19 mice of the experimental groups tumours already appeared within 2–4 weeks, while none appeared in that time in the control group. The most probable explanation of this is that, in the control group, the croton oil treatment was begun before the carcinogen had disappeared from the skin, and the first few paintings with croton oil may have been ineffective.)

DISCUSSION.

In previous investigations (Berenblum, 1941 *a* and *b*) an attempt was made to substitute ordinary, reparative hyperplasia for preneoplastic hyperplasia, by applying croton oil to the mouse's skin prior to treatment with a carcinogen. This did not lead to any shortening of the latent period. On the other hand, when croton oil was applied subsequent to a sub-optimal period (8 weeks) of carcinogenic treatment, a very marked increase in the yield of tumours was obtained. From these experiments, and from analogous evidence in the literature (see reviews by Berenblum, 1944, 1947), it was concluded that (*a*) the preneoplastic hyperplasia constituted a specific entity, biologically distinct from ordinary, reparative hyperplasia, and (*b*) that the change from preneoplastic hyperplasia to wart-formation was a less specific phenomenon which could be brought about by a non-carcinogenic irritant—croton oil—as readily as by continued carcinogenic treatment. The term "*precarcinogenic action*" was used to describe the change from normal to preneoplastic hyperplasia, and "*epicarcinogenic action*" for the change from preneoplastic hyperplasia to wart-formation (while the term "*metacarcinogenic action*" was used to describe the change from warts to malignancy).

The work of Rous and his associates also showed carcinogenesis to be composed of separate and distinct phases (Rous and Kidd, 1941; Mackenzie and Rous, 1941; Friedewald and Rous, 1944 *a* and *b*), but with significant differences in point of detail. They found that tumours in the rabbit could exist for long periods in a "sub-threshold state," requiring additional aid for progressive neoplasia, and concluded (Friedewald and Rous, 1944*a*) that carcinogenesis was composed of an "*Initiating Process*," responsible for the conversion of certain normal cells into latent tumours cells, and a "*Promoting Process*," whereby these latent tumour cells were made to develop into growing tumours.

Tannenbaum (1944) also found evidence of independent stages of carcinogenesis, using caloric restriction of the diet during different periods of carcinogenesis as a method of differentiation.

Finally, there is the evidence of Mottram (1944 *a* and *b*), who used a simplification of the croton oil technique, from which he postulated the existence of three distinct phases in the production of a wart: (i) a "*Sensitizing Factor*," which renders cells hyper-responsive to subsequent treatment, by virtue of a non-specific hyperplasia, (ii) a "*Specific Cellular Reaction*," representing the essential

specific process, and (iii) a "*Developing Factor*," concerned with the actual bringing into being of a visible wart. While the existence of a "Sensitizing Factor" has now been disproved (Berenblum and Shubik, 1947), the other two factors are, in effect, alternative names for the two stages described by the other workers.

The importance of Mottram's contribution to this work lies in the simplification in technique, whereby a single application of a carcinogen, followed by repeated applications of croton oil, serve as a means for tumour production. In the present investigation use was made of this "model experiment" to obtain more precise, quantitative evidence on the mechanisms involved in the stages of carcinogenesis.

When the croton oil treatment was kept constant but different carcinogens, in different concentrations, were used for the initial, single painting, the tumour incidences varied from group to group but the average latent period remained the same. On the other hand, when the initial painting with the carcinogen was kept constant but the croton oil treatment was delayed for different periods, the tumour incidence remained the same but the latent periods varied, corresponding approximately to the lengths of the intervals.

Such results could only have arisen if (*a*) the ultimate number of tumours was predetermined by the single, preliminary action of the carcinogen, (*b*) the "latent tumour cells," produced in the first instance, did, in fact, remain *latent* indefinitely, until stimulated to further activity, and (*c*) the effect of the croton oil treatment was to convert *all* the latent tumour cells into visible tumours.

The initial change is evidently a specific process (since it is induced by carcinogens but not by croton oil or other irritants); it is also an irreversible process (since its effect can be quantitatively demonstrated after as long an interval as 20 weeks); and it is a very rapid process—possibly even an instantaneous reaction (since a single application of a carcinogen is sufficient to initiate the process in a large proportion of the treated animals). In contrast to this, the process involved in the subsequent appearance of the visible wart has a very different mechanism: it is not an instantaneous process (since repeated treatment with the croton oil, or any alternative agent, is required to bring it about); it is not specific (since croton oil, and to a lesser degree, other irritants and wound healing, can produce the effect) and it is not irreversible, at least not in its early developmental stages (as Rous and his associates have shown).

Many implications arise from these conclusions, of which a few call for special comment:

The old conception of "preneoplastic hyperplasia" as an essential, specific forerunner of neoplasia must now be abandoned, and with it the terminology (Berenblum, 1941*a*; 1944; 1947) of "precarcinogenic" and "epicarcinogenic" actions, based on such a conception. The quantitative evidence presented above, considered in conjunction with the qualitative evidence of Rous and his associates, indicates that the initial change is a conversion of a few normal cells into a few latent tumours cells, the latter *lying dormant among the surrounding (hyperplastic) non-neoplastic cells until stimulated to further activity.* The present authors, therefore, favour the adoption of the nomenclature of Friedewald and Rous (1944*a*), using the term "Initiating Process" to describe the irreversible change of normal into latent tumour cells, and the term "Promoting Process" to describe the conversion of latent tumour cells into growing, visible warts.

The question as to whether the Promoting Process is itself divisible into separate stages, will be considered in a later publication.

This novel conception calls for a new orientation in the study of carcinogenesis. It would seem that, on this basis, there is little prospect of deriving any valuable information from the histological or cyto-chemical studies of pre-neoplastic states, since any characteristic features that may exist in the isolated few *latent tumour cells* will, of necessity, be overshadowed by those of the non-neoplastic cells which predominate. Only when such latent tumour cells can be recognized and investigated individually can such methods provide reliable information.

Another permissible conclusion is in regard to the anomaly that has always existed in assessing carcinogenic potencies. It is known from the reliable data of Bryan and Shimkin (1943) concerning the potencies of 1:2:5:6-dibenzanthracene, 20-methylcholanthrene, and 3:4-benzpyrene, for the subcutaneous tissues of the mouse, that the order of carcinogenicity is DBA:MC:BP when judged on the basis of minimal dose-response, but that it is MC:BP:DBA when judged on the basis of average latent period. It appears, from the present results, that there is no real anomaly, since the minimal dose-response is a measure of Initiating action, while the average latent period is a measure of Promoting action. Dibenzanthracene is undoubtedly a potent Initiator, but a weak Promotor; benzpyrene is moderately potent both as Initiator and Promotor; croton oil, on the other hand, is exceptionally potent as a Promotor, but quite useless as an Initiator. When a substance is painted continuously, some confusion may arise as regards these two qualities; but by the use of the "model experiment" the two can be investigated independently.

SUMMARY.

1. In order to study the stages of carcinogenesis by quantitative means, use was made of the technique, based on Mottram's work, whereby tumours of the mouse's skin may be induced by a single application of a carcinogen, followed by repeated applications of croton oil.

2. When the croton oil treatment was kept constant but different carcinogens were used for the initial painting, the tumour incidence varied from group to group but the average latent period remained the same.

3. When the initial painting with the carcinogen was kept constant but the croton oil treatment was delayed, the tumour incidence remained the same but the latent period varied, corresponding approximately to the lengths of the intervals free from treatment.

4. It was concluded that the initial action in carcinogenesis constitutes a sudden and irreversible process, whereby a few normal cells are changed into permanently altered "latent tumour cells," which lie dormant among the non-neoplastic cells. The mechanism by which these latent tumour cells are made to develop into tumours is altogether different from that of the initial transformation.

5. Some of the implications of these conclusions are discussed.

We wish to thank Mr. H. W. Wheal for technical assistance and for the care of the animals.

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EFFECT OF FEEDING 7OH-2-ACETAMINOFLUORENE TO ALBINO RATS.

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A SURVEY of the carcinogenic action of 2-acetaminofluorene (AAF) has been recently published (Bielschowsky, 1947). In 1945 Bielschowsky isolated 7OH 2-acetaminofluorene (7OH-2AAF) from the urine of rats fed AAF. He considered that 7OH-2AAF was not the only metabolite of AAF, but " the derivative which is excreted in the largest quantities in the urine." In experiments of his in which 7OH-2AAF was fed to rats for 62 weeks the substance did not show any carcinogenic activity.

In the work now reported the effect of feeding 7OH-2AAF to albino rats over a period of two years is compared with the effect of feeding AAF.

EXPERIMENTAL.

AAF and 7OH-2AAF were prepared by J. L. Everett and F. Goulden under the direction of Prof. G. A. R. Kon, in the Chester Beatty Research Institute, Royal Cancer Hospital (Free), London. The author wishes to express her indebtedness to these chemists, without whose co-operation the experiments would have been impossible. 7OH-2AAF was prepared as described by Goulden and Kon (1945). The substance was purified by sublimation, which method gives a colourless product more readily than does crystallisation ; the product was sometimes slightly grey. The method of preparation employed precludes contamination with acetaminofluorene.

Albino rats of both sexes, weighing about 100 g. at the beginning of the experiment, were fed on a semisynthetic diet consisting of—

	g. weight.	Percentage of calories.
Starch	1260	39·6
Sugar	630	18·6
Casein	540	12·7
Lard	420	29·1
Cod-liver oil	7	..
Mineral salt mixture (Glaxo)	126	..

The diet was made up in amounts sufficient for about 8 to 10 days. It was supplemented daily with 1 to 2 g. carrots or greens, and about 1 g. "National Bread" of 80 to 82 per cent extraction. Twenty female and fifteen male rats were kept as controls; the same number received AAF or 7OH-2AAF in a concentration of 0·07 per cent. Food and water was allowed *ad lib*. The control rats and rats receiving 7OH-2AAF consumed about 10 g. of food daily; the food intake of rats on AAF was only about 5 to 8 g. The rats were weighed weekly. The growth rate, which was much the same in all three groups, was about 0·5 g. daily. Female rats reached a weight of between 200 and 220 g., and male rats between 250 and 300 g.

At the beginning of the experiment rats were killed at frequent intervals, as the tissues were used in experiments on the succinoxidase activity of livers from rats receiving various carcinogenic and non-carcinogenic compounds. Twelve months after the beginning of the experiment the first tumour appeared in a rat receiving 7OH-2AAF. The surviving rats of these groups (6 females and 2 males) were kept until they showed signs of ill-health. Thus those rats which were allowed to live 24 months consumed approximately 3·1 g. of the compound. In the group receiving AAF no rat survived 461 days. The control experiment was terminated after 500 days.

When rats were killed the organs were removed immediately, weighed and fixed for histological examination.

RESULTS.

(*a*) Rats receiving AAF (Fig. 1): The first tumour appeared on the 183rd day; 83 per cent of the rats surviving beyond that period developed tumours. The localization of these tumours was the same as described by other authors. Sixteen were in the liver, being evenly distributed throughout this organ, and varying in size from 2 to 10 mm. in diameter. Histologically these tumours were mostly hepatomas or cystic or non-cystic cholangiomas; metastases in the lymph glands and lungs were observed. Apart from the hepatic tumours two mammary carcinomas, two uterine epitheliomas, one epithelioma in the external ear and one intestinal adenocarcinoma were found.

(*b*) Rats receiving 7OH-2AAF (Fig. 2): The first tumour appeared on the 380th day. This rat (female) showed a severe one-sided exophthalmos (Fig. 3, 4, 5, 6). Microscopical examination revealed a retrobulbar epithelioma. An adenoma in the lung was also found; the liver was normal. Of the remaining seven rats one

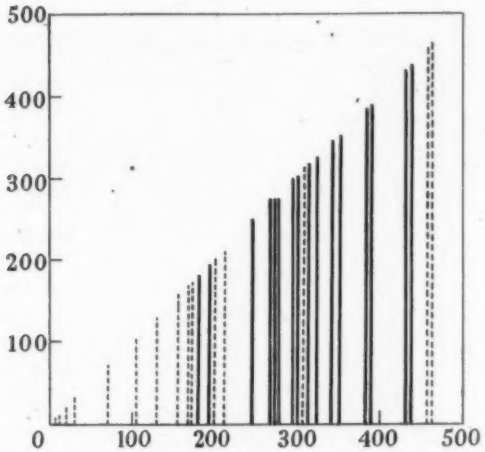


FIG. 1.—Incidence of tumours in rats receiving 2AAF.

FIGS. 1, 2 and 7.—The abscissa and ordinate represents duration of the experiment. Each vertical line represents a rat; the length of the line corresponds to the number of days before the rat was killed.

. . . . . = Rats without tumours.
—.—.—. = Rats with benign tumours.
——— = Rats with malignant or possible malignant tumours.

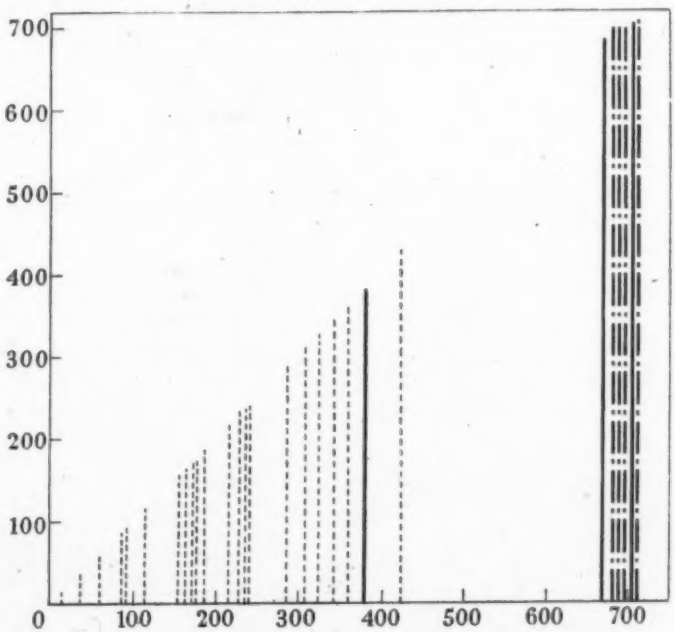


FIG. 2.—Incidence of tumours in rats receiving 7OH-2AAF.

died of pneumonia on the 424th day; no sign of neoplasia was found. The findings in the other rats are summarized below:

	Sex.	Time of killing.	
1.	Female	700 days	Advanced fatty degeneration of the liver. There was a small nodule below the capsule with the appearance of either a hepatoma or a regenerative nodule. Papillomatosis of the stomach. Hyperplastic gland behind the eye with retention of secretion. (Exophthalmos was observed some weeks before the animal was killed.)
2.	,,	685 ,,	Carcinoma of the axillary mamma with retention (milk) cyst. Adenoma in an inguinal mamma very well encapsulated. Epithelial growth in the lung.
3.	,,	700 ,,	Fibroadenoma of mamma. Much irregular growth of the bronchial epithelium. Epithelial growth in the lung most likely basis of chronic pneumonia. No certain malignancy of the liver, but an area where the cells were of a different character from the surrounding ones; this might be the beginning of a hepatoma.
4.	,,	702 ,,	Tumour of mamma; no histological investigation; specimen lost.
5.	Male	705 ,,	Small hepatoma. The kidney shows hyaline casts in the medulla, atrophy of the tubular epithelium and focal lymphocytic infiltration.
6.	,,	705 ,,	Small cholangioma in the liver, nodules of possible hepatoma. Kidney changes similar to those of the other male, but to a lesser degree.

The livers of the four rats showing microscopical changes suggestive of a hepatoma were macroscopically very pale, fatty, and had an uneven surface, due to numerous small nodules about 2 mm. in diameter.

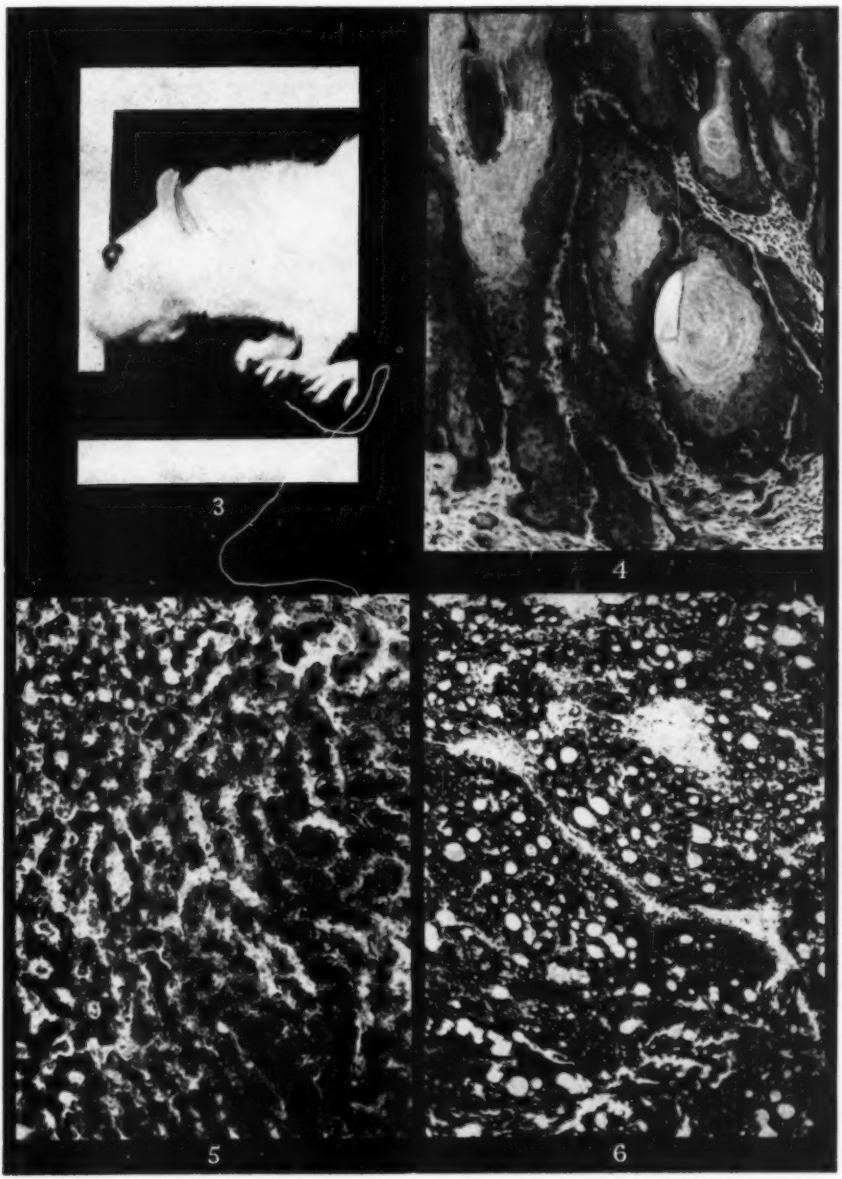
(*c*) Control rats (Fig. 7): In the group of rats killed on the 500th day, one rat with a carcinoma of the small intestines with metastases in the regional lymph gland was observed. Microscopically an incipient cholangioma was found in one rat and a fibroadenoma in another.

DISCUSSION.

The carcinogenic activity of AAF was very much reduced by hydroxylation. The appearance of the tumours was delayed from about 200 days in the animals receiving AAF to about 700 days in the group receiving 7OH-2AAF. As the

DESCRIPTION OF PLATE.

FIG. 3.—Rat showing exophthalmos due to retrobulbar epithelioma.
FIG. 4.—Retrobulbar epithelioma (× 80).
FIG. 5.—Hepatoma (× 140).
FIG. 6.—*Ca.* Mamma (× 76).



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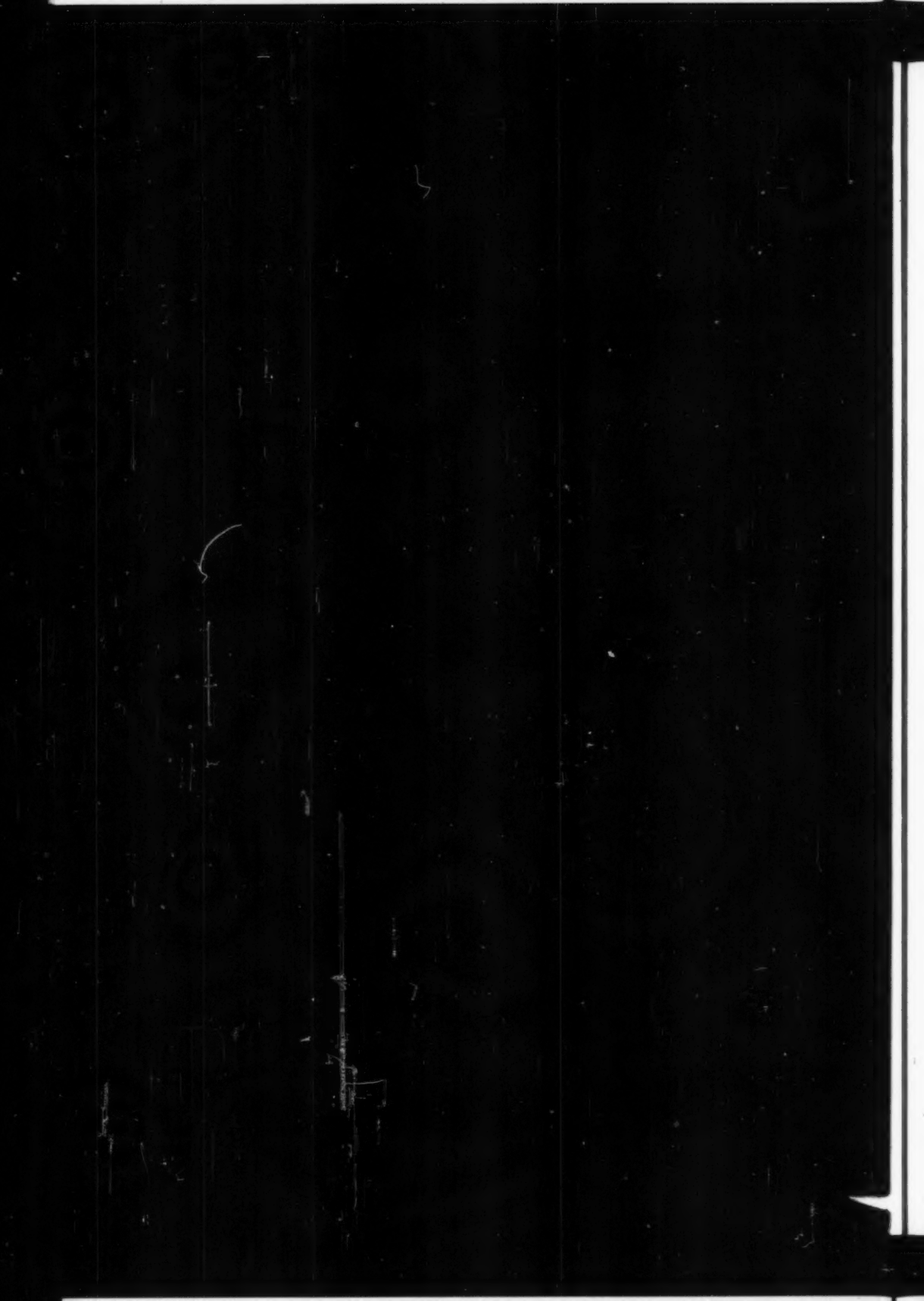

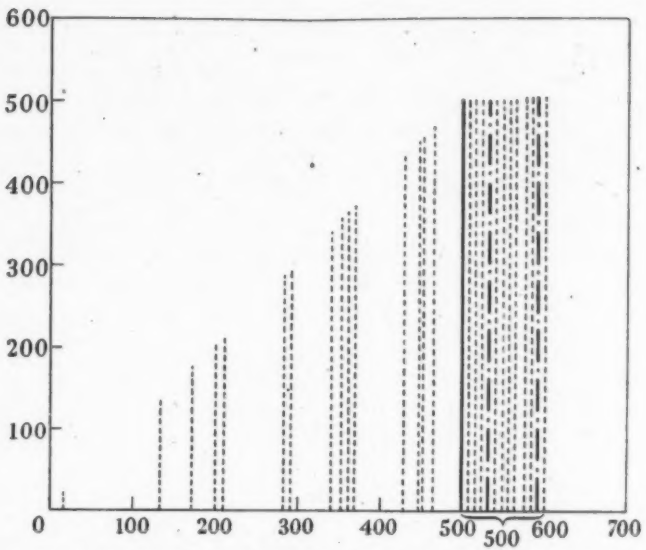

FIG. 7.—Incidence of tumours in control rats. The final group of rats were killed on the 500th and 501st day.

food consumption in the group receiving 7OH-2AAF was greater and the tumours developed later the amount of the compound ingested before the appearance of tumours was about three to four times larger than for AAF. The localization and distribution of the tumours was about the same in the two groups. The control group was not completely tumour-free. The occurrence and distribution of tumours in large colonies of rats has been described by Curtis *et al.* (1931). In their colonies of 3486 rats 426 spontaneous tumours occurred (1·3 per cent). The incidence of tumours increased with the increasing age, and in the age groups comparable with those in the present paper was, at 15 months 1·52 per cent and at 27 months 19·6 per cent. In the present experiment three of the 17 control rats (17 per cent) killed in the 15th month were tumour-bearing. This figure is higher than the highest figure for a strain given by Curtis *et al.* (9·5 per cent in the 15th month), but the significance of this difference is doubtful in view of the small number of animals in the experiments here reported. The age of the rats receiving 7OH-2AAF was about 25 months when they were killed; the number with tumours (7 out of 8) and the type of these tumours makes it improbable that the tumours arose spontaneously.

The finding of three tumours in the control group raises the question of how far the diet employed predisposes to the development of tumours. All animals receiving 7OH-2AAF showed fatty change in the liver long before the appearance of neoplastic changes, and similar fatty change was also observed in a number of control rats. It seems possible that the changes found are the result both of a weak carcinogen and a diet enhancing carcinogenesis.

SUMMARY.

(1) Fifteen out of twenty albino rats receiving 0·07 per cent AAF developed tumours between the 183rd and 461st day of the experiment.

(2) Seven out of eight rats receiving 0·07 per cent 7OH2-AAF for more than a year showed neoplastic changes, one after 380 days and the remainder at about the 700th day.

(3) Three tumours were found in 17 control rats killed after 500 days.

I wish to thank Prof. Sir E. L. Kennaway for constant help and encouragement, and Dr. F. Bielschowsky for his assistance with the histological investigations. My thanks are also due to The British Empire Cancer Campaign for a grant supporting this work.

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THE HISTOLOGY OF PULMONARY TUMOURS OF THE RAT INDUCED BY 2-ACETYLAMINOFLUORENE.

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IN a series of experiments on the carcinogenic action of 2-acetylaminofluorene and related compounds on rats (Bielschowsky, 1944, 1946) tumours have been observed in a variety of anatomical situations. The most frequent sites are the breasts, liver, small intestine, ductus acousticus (external auditory meatus), and lungs. The lung tumours show some points of similarity in structure and origin to those which occur in the mouse, in addition to other features (e.g. squamous metaplasia) which are not seen in the mouse tumours. In view of this, the whole of the available material has been submitted to histological investigation, and the present communication reports the results.

MATERIAL.

The rats in the various experiments belonged to the Wistar and Piebald strains; in one experiment, which showed the highest incidence of tumours, they were Wistar male × Piebald female hybrids. Pulmonary tumours were found in approximately 10 per cent of the experimental animals.

Macroscopically, the pulmonary tumours are not so numerous as those found in mice. Usually there are from 3 to 4 nodules, sometimes only 1 or 2. They are shiny white nodules, most frequently 1 to 2 mm. in diameter, very slightly raised above the surface of the lung. The more malignant tumours, however, can become fairly large (up to 15 mm.), with alteration of the normal configuration of the lobe in which they are situated. This generally happens only in one nodule, the others in the same animal remaining small. On section they are white or yellowish in colour ; if squamous change is present it is sometimes possible to detect a characteristic granularity on the cut surface. They have not been seen in untreated rats. Bronchiectasis occurs in a considerable proportion of older rats, and when extensive might obscure the presence of tumour nodules, so that it is difficult to be quite certain that they never occur spontaneously. The experimental rats were usually killed when less than one year old, however, and at this age bronchiectasis is not very evident. In view of the considerable incidence of squamous tumours in these lungs, it is necessary to mention that many of them occurred in animals without ductus acousticus tumours, or other possible source of squamous metastasis. The lungs of 46 animals in all have been examined microscopically during the present investigation.

RESULTS.

Two main types of histological structure have been found in these tumours, which may be briefly described as tubulo-papillary cuboidal-celled adenomatous, and squamous. Frequently both types of structure are seen in the same nodule, and we believe that they can be traced back to a common origin in the bronchiolar epithelium.

The tubulo-papillary type, which was present in 31 animals, shows alveoli lined by cuboidal or low columnar epithelium, generally arranged in a single continuous layer (Fig. 1, 2, 3). The walls of these alveoli are thrown into folds, over which the epithelial lining is maintained. The stroma appears to consist of the condensed walls of collapsed pulmonary alveoli, as shown by its high content of elastic tissue. This type of tumour is very similar to the papillary cystadenoma of the mouse's lung, the principal differences being the more clear-cut outline of the individual epithelial cells, and the presence of a considerable number of mitoses. The flattened or syncytial types of lining cells are not seen in the rat. Frequently the tumour alveoli contain inflammatory cells, especially macrophages and lymphocytes, less frequently eosinophile and neutrophile polymorphs.

In many of these tumours it has been possible, with serial sections, to demonstrate continuity between the tumour epithelium and the epithelium of a terminal bronchiole (Fig. 4, 5). In such cases the lumen of the bronchiole generally contains inflammatory exudate similar to that seen in the tumour alveoli. In two instances the tumour epithelium shows evidence of mucus secretion.

On the whole, the malignancy of these tumours, as judged by histological criteria, was higher than in the corresponding mouse tumours. One tumour had invaded the wall and lumen of a pulmonary vein. In two animals metastases of the lung tumours were found in the diaphragm and anterior mediastinum. In one of these the secondary deposits showed a vigorous fibroblastic proliferation of the stroma. The primary adenocarcinomata were characterized by

irregular growth, hyperchromasia, frequent mitoses, and columnar cell type; evidence of inflammation within the tumour was not striking.

The squamous type of tumour appears to develop sometimes from the tubulo-papillary type by metaplasia (Fig. 7, 8, 9). A high proportion (about 40 per cent) of the latter show foci of squamous metaplasia, which may come to be the dominant structure of the nodule. In other cases, but less frequently, there has been seen a direct squamous metaplasia of the bronchial epithelium as it enters the tumour. In some of the squamous nodules no evidence of cuboidal epithelium was found, nor could association with the bronchiolar lining be demonstrated. Most of the squamous foci show some degree of keratinization. In many of the squamous nodules the tubular structure is preserved, the alveoli being lined by stratified and keratinized epithelium, and containing macrophages, etc., in addition to desquamated epithelial cells. The average size of the squamous nodules is larger than that of the previous type.

The principal histological difficulty in the case of squamous nodules is to be certain in some instances whether the process is genuinely neoplastic, or the result of aberrant regenerative proliferation. The bronchial epithelium of the rat undergoes squamous metaplasia much more frequently than in other species, and this may sometimes be associated with great irregularity of structure. An example of this is seen in the multiple bronchiectatic lesion which is so common in older rats, and while gross bronchiectasis was absent in these experimental animals, it would not be possible to exclude the participation of incipient stages of such a process. Nevertheless, there is good evidence that the squamous deposits were neoplastic in at least a proportion of cases, and in one with metastases the secondary deposits were squamous-celled carcinoma. Predominantly squamous-celled nodules were found in 13 animals.

With regard to the manner of origin of these pulmonary tumours in the rat, the evidence that they are derived from bronchial epithelium by the invasion of foci of chronic inflammation with collapse is impressive, and is, if anything, more convincing than was found by Orr (1947) for the analogous tumours of the mouse. In several instances it has been possible to demonstrate linear continuity between the epithelium lining a terminal bronchiole and the epithelium investing the folds of the tumour. In some lesions the epithelial invasion is incomplete, so that only part of the nodule shows lepidic structure, the remainder being composed of collapsed pulmonary alveoli with a variable amount of in-

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DESCRIPTION OF PLATES.

FIG. 1.—Tubulo-papillary adenoma. Note trabecular collapse inflammation and emphysema of surrounding lung. × 35.

FIG. 2.—Tubulo-papillary adenoma. × 25.

FIG. 3.—Higher magnification of part of Fig. 2, to show cuboidal cells lining tumour alveoli, which contain macrophages and possibly desquamated epithelial cells. × 105.

FIG. 4.—Adenomatous proliferation of cuboidal epithelium derived from the lining of a bronchiole. × 70.

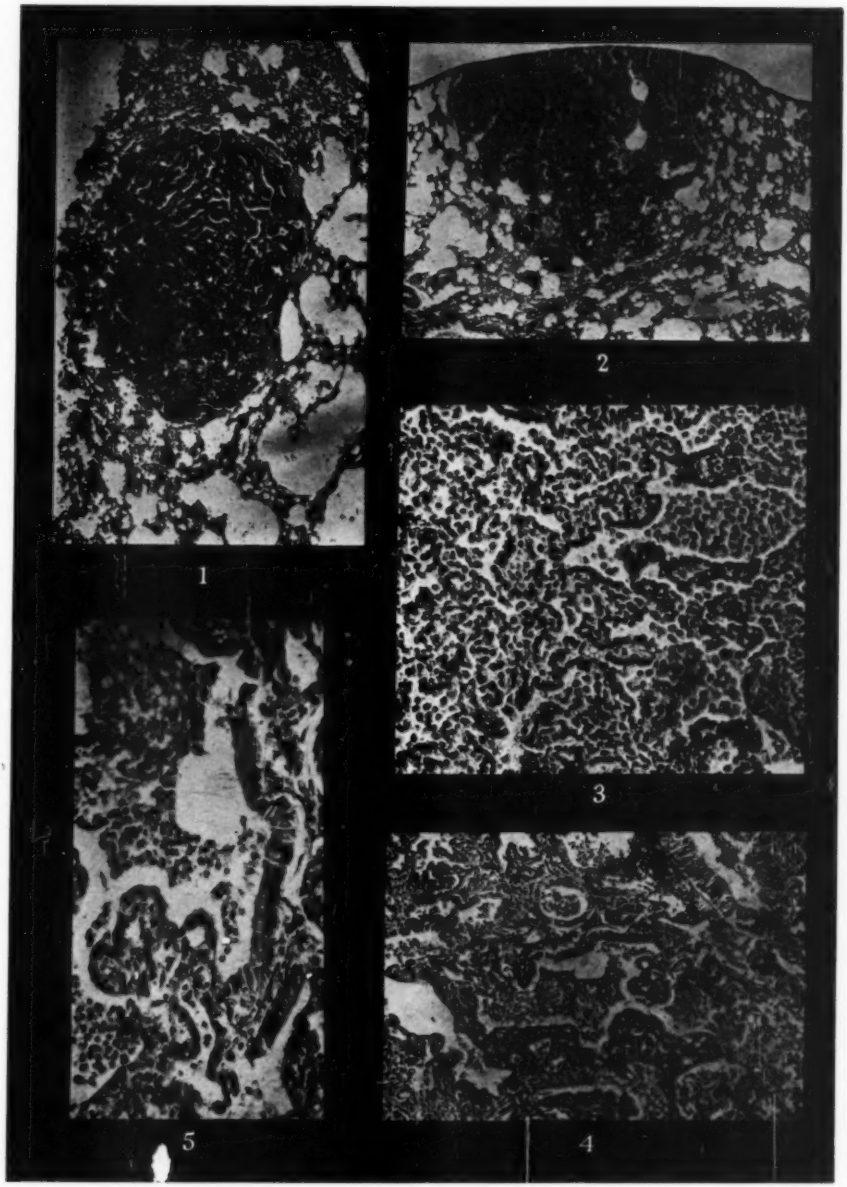
FIG. 5.—Part of Fig. 4 magnified, to show continuity between columnar (bronchial) and cuboidal epithelium. Note also polymorphs and macrophages. × 190.

FIG. 6.—Extension of terminal bronchiolar epithelium in cuboidal form along atria. Neighbouring alveoli contain macrophages, many others collapsed. × 75.

FIG. 7.—Two squamous foci. One is within a bronchus, shows extensive keratinization and foreign body giant cells. The other replaces alveoli. × 35.

FIG. 8.—Squamous carcinoma with keratinization. × 85.

FIG. 9.—Transition from cuboidal to squamous cells within pulmonary tumour. × 140.



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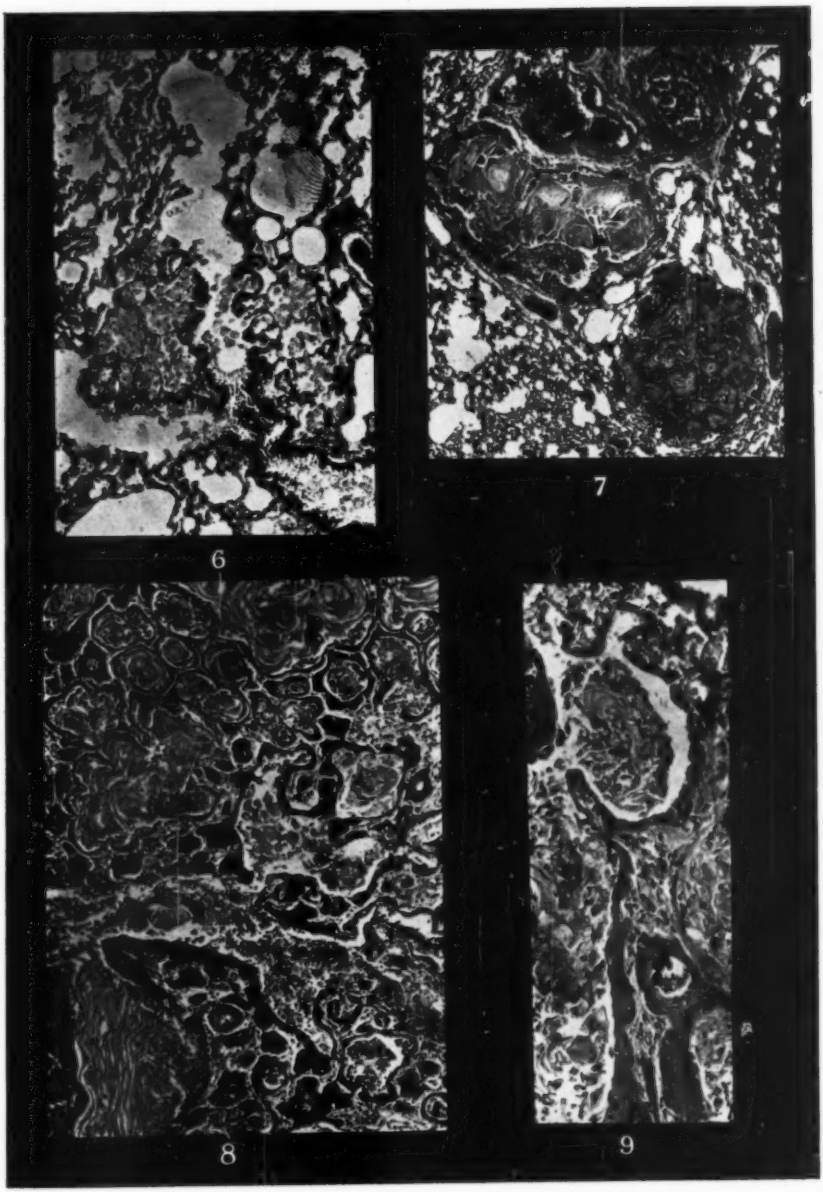

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Orr and Bielschowsky.

flammatory cellular exudate. It is difficult to decide in such circumstances at what point neoplasia may be said to have started, as a complete series could be assembled demonstrating all stages from a purely inflammatory process to a fully established tumour. In other parts of the lung and apart from tumours, there can sometimes be seen prolongation of the bronchial epithelium along the atria; in such circumstances the cells assume the cuboidal form seen in the tumours (Fig. 6). Occasionally papillary infolding of the bronchiolar mucosa is observed. These appearances suggest minor manifestations of the same proliferative process.

Whether or not antecedent inflammation is of aetiological importance, it should be emphasized that some evidence of inflammation has been found in association with every tumour seen. In general, the more completely developed tumours show fewer macrophages, etc., than those in formative stages. Other parts of the lung show trabeculae of collapse infiltrated with lymphocytes, histiocytes, and occasionally eosinophile or neutrophile polymorphs. Perivascular and peribronchial lymphocytic aggregations are common, emphysema is occasional, and fibrosis rare.

COMMENT.

A comparison of the pulmonary tumours induced in rats by feeding 2-acetylaminofluorene with those occurring spontaneously or after treatment with methylcholanthrene or ethyl urethane in the mouse reveals points of similarity and of difference. The fundamental structure of the most usual morphological type of tumour is similar in the mouse and the rat. The rat tumours appear to arise from bronchial epithelium by the invasion of foci of chronic collapse inflammation, as was suggested by Orr (1947) for the histogenesis of mouse tumours. It should be remembered that other theories regarding the manner of origin of the mouse tumours have been put forward, e.g. by Magnus (1939) and Grady and Stewart (1940) While due reservations are necessary when drawing analogies between different species, the present results give a measure of support to Orr's view. The principal differences between the rat and mouse tumours are the pronounced tendency to squamous metaplasia and the higher malignancy of the former. The rat tumours are also more obviously epithelial in origin than those of the mouse.

Jaffé and Jaffé (1947) describe pulmonary adenomata in rats receiving prolonged treatment with ethyl urethane. Their tumours are stated to be mostly of the same type as occur in mice, and they believe them to be derived from metaplastic alveolar epithelium. They mention that " vegetations " of bronchial epithelium were frequently observed, but failed to find any connection between the tumours and the bronchial branches Wilson, De Eds and Cox (1941), in their original paper on the carcinogenic activity of 2-acetylaminofluorene, drew attention to the frequency of inflammatory changes in the lungs; they mention bronchitis, pneumonia, and marked peribronchial lymphoid tissue. Squamous metaplasia was found only in bronchi related to such changes. They are inclined to regard the inflammatory changes as antecedent. They appear to have encountered direct squamous metaplasia of the bronchial epithelium more frequently than we have, but also mention its absence in control animals.

The view that these rat tumours, and the usual types of spontaneous and

induced mouse tumours, are derived from the bronchiolar epithelium may derive some support from the experiments of Horning (1947) with grafts of lung tissue impregnated with methylcholanthrene. He finds no evidence of cellular activity in the alveoli of grafts, and claims that the tumours develop from the bronchial epithelium. The only reference made by him to inflammatory processes is a brief statement about a photomicrograph of an adenocarcinoma "of which some areas are differentiated to form gland-like structures, after containing an exudate of fibrin and leucocytes." The conditions are, of course, very different in the two types of experiment, as the local concentration of carcinogen in the graft is much higher, and the air spaces of the grafted lung tissue are not exposed to aerial contamination. It is not possible at the present stage to evaluate the validity of such a comparison.

SUMMARY.

The histology and histogenesis have been studied of pulmonary tumours occurring in 46 rats treated with 2-acetylaminofluorene and related compounds. The tumours are usually multiple and average 3 to 4 per animal.

The most frequent histological structure is a tubulo-papillary cuboidal-celled adenoma or carcinoma. Squamous tumours are frequently seen, and appear to arise usually by metaplasia of the previous type. Both types of tumour can be traced back to an origin in the bronchiolar epithelium. They appear to start by invasion of areas of collapse inflammation of the pulmonary alveoli by epithelial prolongations from the bronchi.

Metastasis has been seen with both types of tumour.

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SOME STUDIES ON THE ACTION OF URETHANE ON MICE.

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One of the most recent and important additions made to the list of carcinogenic compounds was the discovery by Nettleship, Henshaw and Meyer (1943) that pulmonary tumours (but no other types of cancer) were induced in C_3H and A line mice by intraperitoneal injections of urethane. Subanaesthetic doses of 1 c.c. of a 10 per cent solution per 100 g. body weight of mouse were effective in producing tumours in A mice in less than 4½ months (Henshaw and Meyer, 1944). It was also effective when given by crystal implantation or in the drinking water (Henshaw and Meyer, 1945). Other hypnotics and many substances more or less related in their chemical structure to urethane were ineffective (Larsen, Rhoads and Weed, 1946). Jaffé (1947) found that urethane would produce both lung and liver tumours in rats.

The chemical simplicity of this potent carcinogen compared with others makes it rather remarkable, especially since it is by no means unrelated to many compounds which are normally present in living organisms. The following work was undertaken in order to obtain more information about the methods of action of urethane and the reason for its specificity for lung.

MATERIAL AND METHODS.

Groups of about ten animals of each sex of three inbred lines of mice (CBA, R III and C57), aged about two months, were injected intraperitoneally with 0·25 c.c. of 10 per cent aqueous urethane solution weekly for 14 weeks, as was done by Nettleship, Henshaw and Meyer (1943). This was a fully hypnotic dose. At first the animals were kept five in a cage, virgin and non-breeding. All animals surviving the treatment were killed off either at seven months after the beginning of the treatment for further experimental studies, or at ten months for examination.

At the same time and under identical conditions, 21 male and 20 female CBA mice, also aged about two months, were injected weekly with 0·2 c.c. of a freshly made 1½ per cent solution of veronal (sodium diethylbarbiturate) intraperitoneally for 14 weeks. This was also a hypnotic dose. Seven males and five females of those that survived treatment were killed at seven months and the rest at ten months after beginning the treatment.

RESULTS.

It was known that the incidence of spontaneous lung adenomas varied in the three lines of mice used, though precise figures are not available. However, it can be stated, in general terms, that the RIII line shows about 10 per cent lung tumours after one year, the CBAs rather rarely have any at this age, and none have been found in the C57 line at any age.

A certain amount of intercurrent disease is always present in the mousery in which the experimental animals were kept. One striking result of the injections with urethane was an increase in the susceptibility of the mice to bacterial infections, with the result that while losses due to disease were few in other experiments in the same mousery, the mice treated with urethane suffered a heavy mortality due to infections during the period of treatment. These were of no specific type, enteritis being the most common. All those mice, which were killed when healthy, possessed very small spleens. Both these results could be ascribed to the specific action of urethane on white blood cells described by Paterson *et al.* (1946). The effect of urethane on susceptibility to disease was well shown by comparison with results obtained from the veronal experiment (see Table I).

TABLE I.

Treatment.	Mice.	Numbers died during treatment.	Numbers surviving treatment.
Veronal	CBA males	3	18
,,	,, females	2	18
Urethane	CBA males	5	5
,,	,, females	8	0
,,	RIII males	7	3
,,	,, females	6	4
,,	C57 males	5	4
,,	,, females	4	3

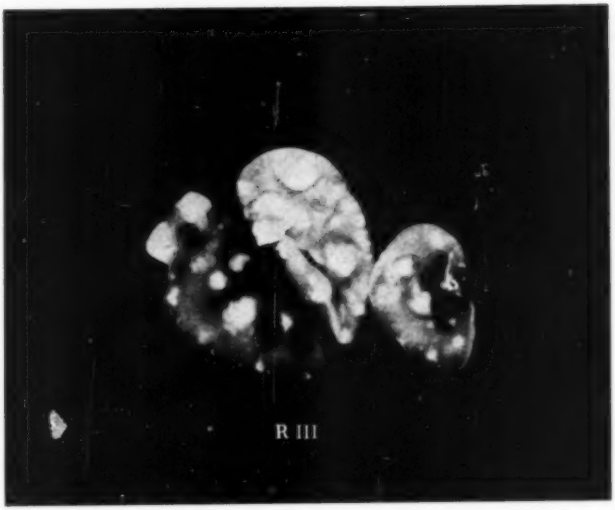
All mice surviving the period of treatment and killed later were apparently free from infection on post-mortem examination, and mortality due to disease ceased completely with cessation of urethane injections.

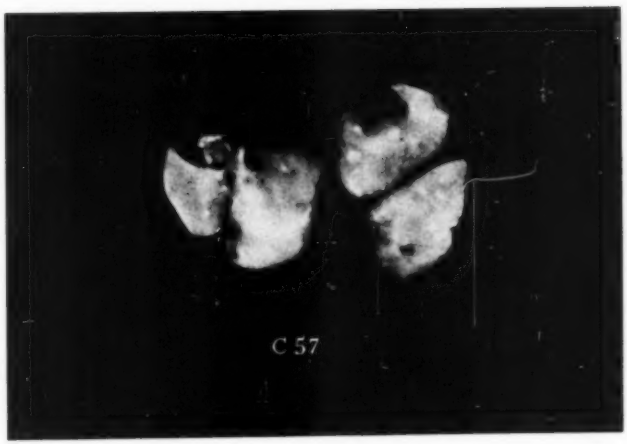
The mice which died of infectious diseases during treatment were usually discovered showing post-mortem changes too far advanced to offer reliable evidence of lung tumours. All the rest of those treated with urethanes however, showed multiple nodules in the lung—very many more than are ever seen in spontaneous cases of pulmonary cancer in mice at this age. On the other hand, of the mice treated with veronal, though a much larger group (of CBA line), only one contained a single adenomatous lump in a lung. This was in an individual killed at seven months after beginning the treatment, and is about the normal expectation in untreated CBAs of this age. Four mice of this group developed single, benign liver tumours, as shown by sections and non-transplantability of one of them. These tumours did not appear to be different from those arising spontaneously in this line, and the number of them produced, although somewhat higher than the average expectation (in contrast to their absence in the few CBAs surviving urethane treatment), does not seem to be significantly related to the treatment.

While all the surviving urethane-treated mice had induced adenomas in their lungs, the number and size of the nodules clearly differed between the different lines, though not between the sexes in the same line. The RIII line suffered

DESCRIPTION OF PLATE.

FIG. 1.—Photograph of the lobes of lungs from C57 and RIII mice after identical treatment with urethane. × 2.


R III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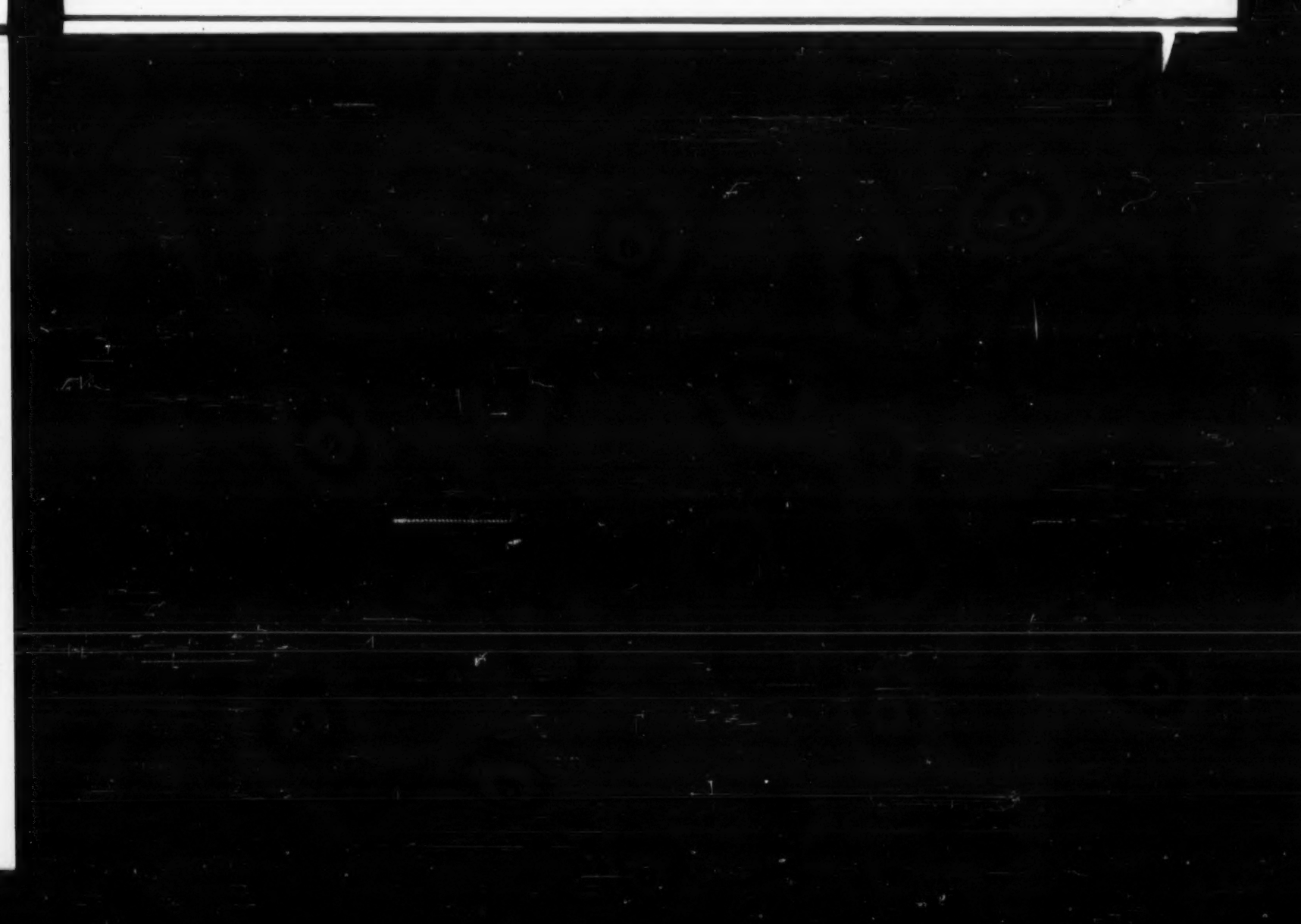

most severely, the lungs being extensively replaced by many large nodules (as well as many smaller ones). The maximum size and the number of nodules was rather less in the CBA line, and much less in both size and number in the C57 mice. This was comparable with the strain differences for susceptibility to spontaneous tumours. The contrast was most impressive in the remaining mice that were killed off at ten months (see Fig. 1)—this was 5½ months after finishing treatment. Only C57s and RIIIs were in existence. The lungs of the C57s showed a small number of tumours, actually smaller in size than was the case in the C57s killed off three months earlier. It would appear a possibility that the C57 lung tumours tended to regress when the treatment stopped. The RIIIs had far more numerous and larger tumours than had the C57s. Whereas the largest C57 tumours were about 2 mm. in diameter, the largest RIII tumours consisted of nodules 7 mm. in diameter; furthermore, the RIII lungs contained about 5–10 times as many lung tumours as the C57s had.

Histologically the tumours consisted of large dark cells apparently derived from bronchial alveolar cells, closely resembling the structure of the tumours studied by Nettleship, Henshaw and Meyer (1943). Mitoses were seldom found, and the tumours appeared benign by histological criteria. The benign nature was also indicated by the results obtained with transplants. For this, the entire lung of a CBA with well-developed tumours was finely minced and injected into twelve young CBA mice, and the same done with an RIII lung, using RIII mice. No growths were obtained after three months.

The heavy increase in susceptibility to disease in the urethane experiment recalls the description of the pneumonia-like lesions sometimes found in the lungs of the mice described by Nettleship, Henshaw and Meyer (1943). These authors pointed out that these lesions are suggestive of virus-type pneumonias, and it is thus possible that the urethane, as a carcinogen, might act through a virus-type mechanism. The considerable decrease in resistance to other diseases found in this experiment seems significant in this direction.

It seemed that the most obvious beginning would be to test for the L or pleuropneumonia-like organisms which often occur in the lungs of rodents (Topley and Wilson, 1946). Facilities for such investigations not being available here, Dr. Klieneberger-Nobel very generously undertook to search for these organisms at the Lister Institute, London. No pleuropneumonia-like organisms were found in the lungs of normal CBAs, but after insufflation of sterile broth into the nostrils of the mice the L-type organisms were recovered. Three mice, two CBAs and one RIII sent seven months after the beginning of treatment with urethane, contained many adenomatous-type lesions, but no pleuropneumonia-like organisms could be recovered from them.

Many materials which act as therapeutic agents in cancer, such as X-rays, radium and the nitrogen mustards, cause sterility, and some cancer inciters, such as X-rays, ultra-violet light, and the hydrocarbons methylcholanthrene and dibenzanthracene also cause defective gametogenesis. Since urethane is both a cancer inciter and has a therapeutic effect on some cancers (Paterson *et al.*, 1946), in addition to causing a leukopenia rather like X-rays (Henshaw, 1944), the following study was undertaken: Six C57 male mice, which had received two weekly injections of 0·2 c.c. of a 10 per cent solution of urethane for an experiment about three months previously, were given three more weekly injections at the same dosage level. After the third injection three of the mice

were killed and their testes sectioned, and each of the other three were placed in a cage with three adult female mice. The males readily sired large litters and gave not the slightest indication of sterility, nor was any interference or breakdown of gametogenesis noted in the testes examined histologically.

The possibility of urethane acting as a growth promoter, specific or otherwise, on embryonic tissues was investigated. Twenty CBA males were injected subcutaneously with a suspension of minced CBA embryo in normal saline. Ten of these were given weekly injections of 0·2 c.c. of 10 per cent solution of urethane intraperitoneally for two months. Four of the treated animals and three of the untreated animals grew detectable embryomas, all of which regressed by the end of the two months. No specific effect on the embryonic lung was thus detected.

DISCUSSION.

The first experiments with urethane by Nettleship, Henshaw and Meyer (1944) were carried out on C_3H and A mice, both of which, especially the latter, have a relatively high spontaneous incidence of lung cancer. It is clear from their descriptions that the A line responded more readily to the carcinogenic action of urethane. The present work extends this result to three other lines, and the same parallelism between the incidence of spontaneous and induced cancer is found. This suggests very strongly that the urethane merely accentuates a process that normally occurs in mice, and that its action is controlled by the same mechanisms as those controlling normal adenomatous formation in the lungs of mice. The chemical relationship of urethane to known normal constituents of the mammalian organism suggests either that the urethane can substitute for them, perhaps by conversion into a normal constituent, or even that these might be converted to urethane by normal or abnormal metabolism.

As was shown by Larsen, Rhoads and Weed (1946), other hypnotics derived from urea-type structure and related to pyrimidine (in this case veronal) do not have this action. It might be pointed out that they do not have the leukopenia producing action of urethane either. If these two actions are related, then a possible activation of a virus may be considered. This hypothesis avoids the difficulty of having to explain why direct action of urethane on the lung causes only a very minute fraction of cells to become cancerous. It was, therefore, natural to try first to detect the pleuropneumonia-like organisms which are so frequently found in the rodent lung, especially since Nettleship, Henshaw and Meyer (1943) noted a concomitant appearance of pneumonia-like lesons in the lungs of treated mice. The complete absence of these organisms in heavily cancerous lung appears to rule this out as a possibility. It might be pointed out that the apparent lack of response of embryonic tissue in embryomas growing in adults, which, presumably, will not suffer infection by inhaled viruses, lends a little support to the virus hypothesis, though it is, of course, readily agreed that other explanations could account for this negative result.

The rather disastrous effects of many cancer-therapy processes, such as X-rays and mustard gas, upon the sex organs, taken with the effect of urethane on cell division when given in near lethal doses described by Dustin (1947), impelled a check on the effect of ordinary anaesthetic, carcinogenic and therapeutic doses on the testes. It is reassuring that no deleterious action was observed.

SUMMARY.

1. The number and size of lung adenomas produced in RIII, CBA and C57 mice by urethane decreased in that order. This is the same order as the incidence of spontaneous lung cancer.

2. Compared with a group given similar treatment with veronal (which was non-carcinogenic), the urethane-treated animals showed a high incidence of infectious diseases.

3. No pleuropneumonia-like organisms were present in the lungs of the urethane-treated mice.

4. Treatment with urethane did not cause male sterility.

5. No action on embryonic growth was noted.

To Mr. G. R. Knight, for the photograph, the author expresses his appreciation.

All expenses in connection with this work were borne by the British Empire Cancer Campaign.

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THE COURSE OF THE METABOLISM OF BENZPYRENE IN THE SKIN OF THE MOUSE.

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PREVIOUS work has shown that the elimination of benzpyrene from the animal body is by way of a series of chemical changes (Weigert and Mottram, 1946). The available evidence suggests that the whole course of the changes from the benzpyrene to the excretion products—in the case of mice, 8-OH benzpyrene—does not take place at one point in the body.

With this as starting-point an attempt has been made to trace the course of the metabolism at one site. The site chosen for consideration was the skin—as this is one at which tumour formation occurs at the point of application of the carcinogen.

MATERIALS AND METHODS.

The animals used throughout these experiments were Strong A mice aged 8–10 weeks. In every case the experimental work was confined to consideration of an area of 4 square cm. on the flank. The 3:4 benzpyrene was applied as a solution in acetone through a stencil, as described by Calcutt and Powell (1947). Prior to application of the carcinogen the fur was removed by clipping.

At selected intervals after painting the mice were killed and the treated areas of skin excised, minced and processed for extraction of the metabolites. Quantitative estimations were made of the metabolites and the residual unchanged benzpyrene. The methods used for the separations and the estimations were those proposed by Weigert (1947).

It has been shown by Calcutt and Powell (1947) that mice which have been painted, very rapidly lick the reagent concerned off the skin. So for comparison a number of animals were painted and fixed to a board with adhesive tape. Under these conditions they could be maintained fairly well for periods of up to five hours. Estimations were carried out at intervals in the same manner as before.

Additional to these *in vivo* experiments, several series were also carried out using the *in vitro* technique, previously described by Weigert, Calcutt and Powell (1946). By this method measured areas of skin were removed from freshly killed mice, painted with benzpyrene and floated underside downwards on Ringer-Locke solution in Petri dishes. These were incubated at 37°C., specimens being taken at intervals for estimation.

RESULTS.

The general course of the metabolism was followed qualitatively over periods of up to 24 hours. Separation of the acetone extracts of the skins was carried out by both chromatographic and partition methods. Apart from unchanged benzpyrene the only other compound found to be present was the one labelled by Weigert and Mottram (1946) as BPX_2. This was identified from its fluorescence spectrum. No traces of the further metabolic products BPF_1 or BPF_2 could be found.

Quantitative estimates were undertaken of the rate of formation of the BPX_2. Results for the *in vivo* paintings of free and fixed animals are shown in Fig. 1 and 2. In the case of the *in vitro* technique, a series of results was obtained using varying concentrations of the 3:4 benzpyrene. These results are given in Fig. 3–8. Examinations of the Ringer-Locke solutions used in these experiments failed to show anything in the way of diffused metabolites.

DISCUSSION.

Consideration of the data shown above indicates three important findings. These are :

The only metabolite found is BPX_2.

There is an initial time lag in the production of the metabolite.

There is a fairly rapid build-up in the amount of metabolite after the initial delay period, followed by a slow falling off in the amount present.

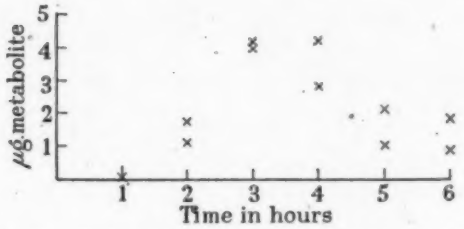

FIG. 1.—Free animals. Each painted with 0·3 mg. benzpyrene in 0·1 c.c. of acetone.

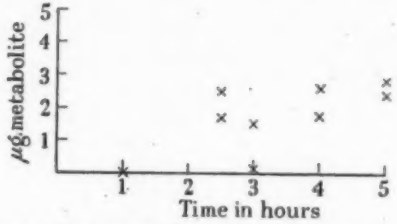

FIG. 2.—Fixed animals. Each painted with 0·3 mg. benzpyrene in 0·1 c.c. of acetone.

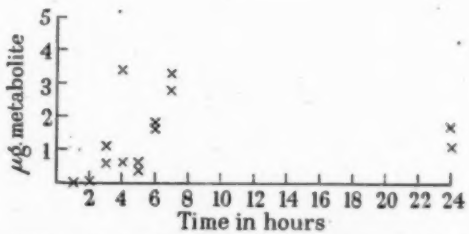

FIG. 3.—Detached skins. Each painted with 0·3 mg. benzpyrene in 0·1 c.c. of acetone.

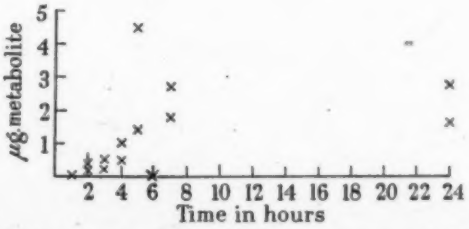

FIG. 4.—Detached skins. Each painted with 0·1 mg. benzpyrene in 0·1 c.c. of acetone.

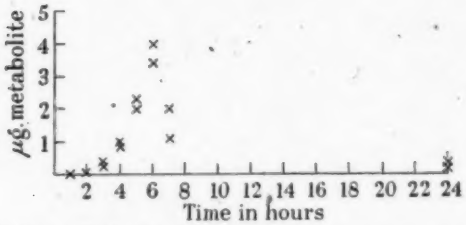

FIG. 5.—Detached skins. Each painted with 0·05 mg. benzpyrene in 0·1 c.c. of acetone.

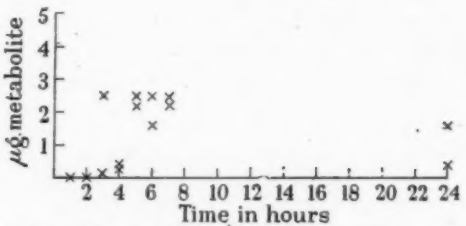

FIG. 6.—Detached skins. Each painted with 0·01 mg. benzpyrene in 0·1 c.c. of acetone.

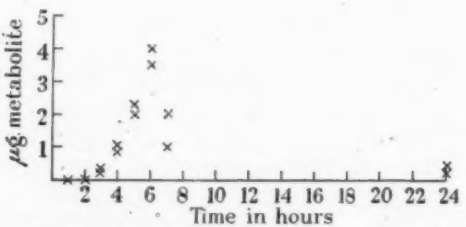

FIG. 7.—Detached skins. Each painted with 0·005 mg. benzpyrene in 0·1 c.c. of acetone.

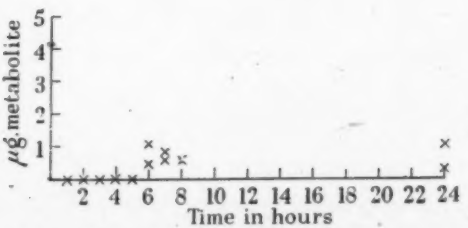

FIG. 8.—Detached skins. Each painted with 0·002 mg. benzpyrene in 0·1 c.c. of acetone.

The fact that BPX_2 was the only metabolite found suggests that the further breakdown products, BPF_1 and BPF_2, are not formed at the site of application of the benzpyrene. This, however, is not positively proved, as the experiments may not have been continued sufficiently long for the formation to occur. Since, however, this change occurs rapidly in other parts of the body (Weigert and Mottram, 1946), it would seem unlikely that there would be any great delay if the process occurred in the skin. Under these circumstances it appears that the benzpyrene is converted to the water-soluble BPX_2 in the skin, but that further conversion to the final excretion products takes place elsewhere in the body. If this is so it follows—assuming a causal relationship between metabolism and carcinogenesis—that the carcinogenic action of the benzpyrene in the skin is exercised by the benzpyrene itself, by the BPX_2 or as a result of the formation of the BPX_2 from the benzpyrene. The compounds BPF_1 and BPF_2 must then be considered solely as excretion products, and as having no connection whatever with the actual process of carcinogenesis.

Estimations of metabolites after intravenous injections of benzpyrene into mice showed a preponderance of BPX_1 (Weigert and Mottram, 1946). On the other hand, when the benzpyrene was absorbed slowly as after intraperitoneal injection, BPX_2 formed the bulk of the first metabolic products. This, considered together with the findings in respect of skin, suggests that BPX_1 is formed when relatively large amounts of the benzpyrene are rapidly brought into contact with tissues, but that BPX_2 is formed when the benzpyrene is slowly introduced. The production of one or the other is thus a function of the physical conditions operative at the time of the introduction of the parent hydrocarbon into the tissues.

The initial time lag before any production of metabolites can be traced is probably of purely mechanical origin, in that time is necessary for the benzpyrene to pass through the skin to the site at which metabolic conversion takes place. However, the possibility of some—as yet undiscovered—preliminary reaction occurring cannot be excluded, though there is no evidence for such an occurrence. Pending further experimental work the exact cause and significance of this time lag phenomenon must remain as unanswered questions.

Once the production of the metabolite has commenced, there is a fairly rapid build-up to a peak value and then a slow decline. Since variation in the amount of benzpyrene applied has little effect on this general pattern of behaviour, it appears that the decline is not a function of the amount of residual benzpyrene present in the unchanged state. The limitation then must be imposed by some other factor. The most likely appears to be the using up of the available cellular substrate involved in the formation of the BPX_2. This view is, however, still open to doubt.

The fact that no metabolic products other than BPX_2 were found does not signify that the decline in the amount of the BPX_2 is not due to its conversion into other compounds. The technique used may not have been suitable or sufficiently delicate to determine this point. In any event it can be said that the loss of the BPX_2 from the skin is a relatively slow process as compared to its formation.

Till the relationship between the metabolism and carcinogenicity of benzpyrene is determined, no assessment of the significance of the points discussed above can be of anything more than academic interest.

SUMMARY.

1. Experiments are described in which the determination of the course of metabolism of 3:4 benzpyrene in mouse skin has been followed both quantitatively and qualitatively over periods of up to 24 hours.

2. The only metabolic product discovered was BPX_2.

3. There is an initial time lag of approximately 1 hour before any metabolite can be found.

4. Once metabolism commences there is a fairly rapid build-up of the metabolite.

5. This build-up reaches a peak value, and then there is a slow decline in the amount present.

6. The meaning and significance of these findings is discussed.

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THE EFFECT OF LIPOID SOLVENTS ON THE RATE OF ELIMINATION AND THE CARCINOGENIC POTENCY OF 3:4-BENZPYRENE AFTER SUBCUTANEOUS INJECTION IN MICE.

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ALTHOUGH the fact is now well established that the incidence and latent period of tumours induced by 3:4-benzpyrene depend to some extent on the solvent employed, a proper understanding of this " solvent effect " is still lacking. This is partly due to the complex nature of the natural oils and fats most frequently used in studies on solvent effects. Their action is the resultant of a multiplicity of component effects which may be either synergistic or antagonistic. Progress is more likely to come from a study of simple synthetic or near-synthetic solvent systems which are more amenable to interpretation in terms of biochemical reactions or properties.

The idea that the pro- or anticarcinogenic effects of solvents might be correlated with differences in the elimination rate of benzpyrene was first formulated by Peacock and Beck (1938) who, on the basis of qualitative visual inspec-

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tion, attributed the anticarcinogenic effect of certain solvents to the faster disappearance of benzpyrene from the injection site. A systematic analysis in which an accurate method for the quantitative estimation of benzpyrene was used failed to establish a clear-cut correlation in the case of some natural lipids, i.e. cod liver oil, mouse fat and their hydrogenation products (Weil-Malherbe and Dickens, 1944; Dickens and Weil-Malherbe, 1946). Likewise, Rusch, Mueller and Kline (1945) could not find any obvious relationship between the rate of elimination of benzpyrene and the solvent effect. On the other hand, Weil-Malherbe and Dickens (1946), when investigating the effects of tricaprylin solutions of cholesterol and phospholipids, obtained the unexpected result that the more rapid elimination of benzpyrene was associated with the higher carcinogenic activity and the slower elimination with lower carcinogenic activity. That a correlation was obtained at all was probably due to the comparative simplicity of the solvent systems used, but confirmatory evidence is clearly desirable before the results can be generalized. It was with this object in view that a study of the effects of a number of different compounds on the elimination rate of benzpyrene was undertaken.

MATERIALS AND METHODS.

Phospholipids.—Phospholipids were prepared from ox brain by the method previously described (Weil-Malherbe and Dickens, 1946), with the difference that the preparation was done on a larger scale, 2300 g. of brain being used as starting material. The alcohol-soluble (lecithin) fraction weighed 22·5 g. and contained 3·12 per cent P and 2·50 per cent N, whereas 24 g. of the alcohol-insoluble (kephalin) fraction contained 3·78 per cent P and 2·34 per cent N. Both fractions were kept in sealed ampoules under nitrogen. The high N-content and, in the case of lecithin, the low P-content indicate that the fractions were still very impure.

Other compounds.—The following preparations were carried out:

Perbenzoic acid (*Organic Syntheses*, 1933). A 15 per cent solution in tricaprylin was prepared and kept in the refrigerator. The solution was standardized iodometrically before use.

Tricaprylin (Hartwell, 1940). M.P. 8·5–10·5°.

Ethyl linoleate (*Organic Syntheses*, 1942).

Ascorbyl palmitate (Swern, Stirton, Turer and Wells, 1943). M.P. 115°. 0·1756 g. used 7·80 ml. 0·1 *N* iodine solution.

Cholestanol (*Organic Syntheses*, 1937). M.P. 141°. Liebermann-Burchard reaction negative.

7-Dehydrocholesterol (Windaus, Lettré and Schenck, 1935; Rosenberg, 1941). M.P. 143°.

Ergosterol peroxide (Windaus and Brunken, 1928). M.P. 174°.

All melting points are uncorrected.

Coprostanol, *epi*-coprostanol and *epi*-cholestanol were supplied by Dr. H. King, F.R.S., National Institute for Medical Research.

Other chemicals used were commercial products of various origin.

Benzpyrene was estimated as previously described (Weil-Malherbe, 1944; Dickens and Weil-Malherbe, 1946).

1. *Oxidation of* 3:4-*Benzpyrene* in vitro.

It has been suggested (Weil-Malherbe and Dickens, 1946) that the retardation of benzpyrene elimination and the delay of tumour formation caused by phospholipids might be connected with their antioxidant properties. It was, however, realized from the beginning that antioxidant activity in the accepted sense cannot be expected to occur in the living animal cell. Antioxidant activity probably consists in the breaking of reaction chains initiated by autoxidation processes (e.g. Filer, Mattil and Longenecker, 1944). The existence in the reduced form of autoxidizable substances, such as ascorbic acid, thiol compounds, etc., in the living cell is symptomatic of the absence of widespread autoxidation processes, and reaction chains of any considerable length are unlikely to occur in the complex medium of the cell. It is therefore not surprising that Rusch, Mueller and Kline (1945) failed to find any correlation between the autoxidizability of a solvent and its effect on carcinogenesis.

On the other hand, carcinogenic hydrocarbons, such as 20-methylcholanthrene (Simpson and Cramer, 1945) or 3:4-benzpyrene (Weigert and Mottram, 1946) seem to be oxidized as soon as they permeate into the interior of almost any living cells with which they come into contact. Their oxidation is therefore more likely to be incidental to the normal metabolic activities of the cell than to the action of a specific enzyme. Such a mechanism might involve a peroxide or a radical of high oxidant potential with or without the intervention of a peroxidase. Such an induced oxidation might be inhibited by phospholipids or other antioxidants owing to their higher affinity for the oxidant.

It was therefore felt that useful information might perhaps be gathered from a study of the oxidation of benzpyrene *in vitro* and the action of various antioxidants and combinations of antioxidants thereon.

TABLE I.—*Oxidation of Benzpyrene by Hydrogen Peroxide in* 80 *per cent Acetic Acid.*

Each tube contained 1 mg. benzpyrene in 10 ml. 80 per cent acetic acid. After addition of 0·2 ml. 30 per cent hydrogen peroxide the tubes were left at room temperature for 16 hours.

Additions.	Percentage of benzpyrene present at end of experiment.
0	88·6
200 mg. lecithin	56·4
200 ,, kephalin	56·4
200 ,, lecithin + 1 mg. ascorbic acid	57·5
200 ,, ,, + 1 ,, α-tocopherol (Roche)	65·2
200 ,, ,, + 1 ,, ascorbic acid + 1 mg. α-tocopherol	60·8

As shown by Table I, benzpyrene is slowly oxidized by a solution of 0·6 per cent hydrogen peroxide in 80 per cent acetic acid. In this system both lecithin and kephalin had a powerful pro-oxidant effect which was little modified by their combination with other antioxidants.

An effect in the opposite direction was found when the oxidation of benzpyrene was carried out in a lipoid medium. In these experiments a solution of

perbenzoic acid in tricaprylin was added to a tricaprylin solution of benzpyrene (Table II). Here the addition of lecithin resulted in a marked inhibition of the oxidation, which was still further enhanced by combination with α-tocopherol, but was reduced by combination with ascorbic acid or carotene. Almost the maximum potentiation effect is observed with a concentration of 0·1 mg. tocopherol in 2 ml. (Table III). Cholesterol was found to have an effect similar to, though much smaller than, that of lecithin (Table IV). This effect is somewhat accentuated by carotene, but reduced by ascorbic acid.

TABLE II.—*Oxidation of Benzpyrene by Perbenzoic Acid in Tricaprylin.*

Each tube contained 1 mg. benzpyrene in 1·5 ml. tricaprylin. After addition of 0·5 ml. of a 12 per cent solution of perbenzoic acid in tricaprylin the tubes were sealed under nitrogen and left at room temperature for 70 hours in the dark.

Additions.	Percentage of unchanged benzpyrene.
0	14·9
30 mg. lecithin	45·5
30 „ „ + 0·5 mg. α-tocopherol (Roche)	52·2
30 „ „ + 0·5 „ ascorbic acid	31·4
30 „ „ + 0·5 „ α-tocopherol + 0·5 mg. ascorbic acid	33·8
3 „ β-carotene (Roche)	15·1
30 „ lecithin + 3 mg. β-carotene	26·8
30 „ „ + 3 „ „ + 0·5 mg. α-tocopherol	28·2

TABLE III.—*Effect of Combination of Lecithin with Varying Amounts of α-Tocopherol on Oxidation of Benzpyrene by Perbenzoic Acid.*

Arrangement as in preceding experiment (Table II).

Additions.	Percentage of unchanged benzpyrene.
30 mg. lecithin	31·2
30 „ „ + 0·1 mg. α-tocopherol	47·5
30 „ „ + 1 „ „	55·0
30 „ „ + 10 „ „	55·5

TABLE IV.—*Effect of Cholesterol on Oxidation of Benzpyrene by Perbenzoic Acid.*

Each tube contained 1 mg. benzpyrene in 1·5 ml. tricaprylin. After addition of 0·25 ml. of a 5 per cent solution of perbenzoic acid in tricaprylin the tubes were sealed under nitrogen and left at room temperature for 48 hours in the dark.

Additions.	Percentage of unchanged benzpyrene.
0	33·8
30 mg. cholesterol	48·1
30 „ „ + 3 mg. carotene	56·0
30 „ „ + 3 „ ascorbic acid	41·9

At the time these experiments were performed it was not known that the effects of the new phospholipid samples differed from those of the earlier sample in experiments *in vivo*. The fact that cholesterol and phospholipids had similar effects *in vitro* and seemingly opposite ones *in vivo* suggested that the model systems did not approximate closely enough to *in vivo* conditions. The experiments were therefore discontinued; they do show, however, that the oxidation of benzpyrene may be affected by other substances present in concentrations which were previously shown to be effective in experiments *in vivo*.

II. *The Effect of Lipoid Solvents on the Rate of Elimination of 3:4-Benzpyrene after Subcutaneous Injection in Mice.*

Whereas their antioxidant properties were thought to be responsible for the effects of phospholipids on the elimination rate and carcinogenicity of benzpyrene, it was also tentatively suggested that the opposite effects of cholesterol might be connected with the well-known propensity of sterols and steroids to form coordination compounds. By investigating a number of substances belonging to the two classes of antioxidants and sterols as to their effect on the elimination rate of benzpyrene, it was hoped to obtain further evidence by which the reality of the proposed mechanisms might be examined. Since synergistic effects are not uncommon when two or more antioxidants are present together (Mattill, 1945) various such combinations were tried.

The substances to be tested were dissolved in tricaprylin containing 0·1 per cent benzpyrene and injected into batches of 5–10 mice. After an interval of 4–5 weeks the mice were killed and the remainder of benzpyrene estimated. Since it has been shown by ourselves (Dickens and Weil-Malherbe, 1946; Weil-Malherbe and Dickens, 1946) and others (Berenblum and Schoental, 1942) that the elimination of benzpyrene follows roughly the course of a monomolecular reaction, the reaction constant may be determined from a single observation. A period of 4–5 weeks was found to give the most favourable results, as the benzpyrene level has decreased to a significant extent, but is usually still sufficiently high to allow of accurate measurement.

Male mice of the Glaxo FF strain were used. Each mouse received 0·3 mg. benzpyrene, dissolved in 0·3 ml., subcutaneously into the left axillary region. The animals were examined in ultraviolet light for leakage of fluorescent material immediately after the injection and thereafter several times weekly, and all those showing signs of leakage or ulceration were rejected. Ulceration was only observed with a few of the solutions, most of them being well tolerated.

Tricaprylin solutions containing 0·6 mg. of β-carotene (Roche) proved extremely toxic for mice after subcutaneous injection, leading to the death of 13 out of 16 animals after 2–5 days. No mention of this high toxicity of subcutaneously administered β-carotene for mice could be found in the literature. Sexton, Mehl and Deuel (1946) injected 0·3 mg. carotene in oil intravenously, and up to 1·1 mg. intraperitoneally, into rats, apparently without ill-effects. Chauchard (1941) reported that intraperitoneal injection of carotene has a depressant action on nervous response in rats, guinea-pigs and rabbits.

Results.—The monomolecular reaction constants were calculated according to the formula $k = \frac{1}{t} \log \frac{a}{S}$ where t = days after injection, a = initial and

S = final quantity of benzpyrene. The mean value of k and the standard error of the mean were calculated for each batch. The figures are assembled in Tables V and VI. A series of 24 analyses on mice injected with benzpyrene solutions in pure tricaprylin were carried out to provide a standard of comparison. The mean of k was found to be $0{\cdot}0154 \pm 0{\cdot}00132$, a value in excellent agreement with that previously obtained (Weil-Malherbe and Dickens, 1946).

The mean k-value of each experiment was compared with the mean k-value of the standard series and the difference was tested for statistical significance. It appears from Table V that out of all the antioxidants and combinations of antioxidants tested only one had a delaying effect of probable significance on benzpyrene elimination, viz. ascorbyl palmitate in a concentration of 2 per cent. No effect was observed with the same substance in a concentration of 0·5 per cent. Unfortunately ascorbyl palmitate caused ulceration in a number

TABLE V.—*Elimination of Benzpyrene from Solutions Containing Antioxidants and Related Substances after Subcutaneous Injection into Mice.*

Addition in tricaprylin.	Concentration (%)	Total number of animals.	k (mean).	Standard error.	P.*
Lecithin	3	8	0·0242	0·00228	<0·01
Kephalin	3	5	0·0251	0·00588	0·02
α-Tocopherol	0·3	5	0·0291	0·0056	<0·01
	1	5	0·0442	0·0107	<0·01
Ascorbyl palmitate	0·5 (ulc.)	7	0·0148	0·00295	n.s.
	2 ,,	9	0·0091	0·0028	0·03
Ethyl linoleate	3	5	0·0132	0·0023	n.s.
	100	6	0·0251	0·0071	0·03
Ethyl chlorophyllide	0·3	5	0·0218	0·00464	n.s.
2-Methyl-3:4-naphthoquinone	0·3 (ulc.)	3	0·0272	0·0043	—
β-Carotene	0·2	2	0·0132	—	—
Lecithin (3%) + α-tocopherol (0·3%)		4	0·0373	0·00106	<0·01
Kephalin (3%) + α-tocopherol (1%)		5	0·0431	0·00563	<0·01
Cholesterol (3%) + α-tocopherol (1%)		5	0·0287	0·00436	<0·01
Lecithin (3%) + ascorbyl palmitate (0·5%)		5	0·0104	0·00231	n.s.
Lecithin (3%) + ascorbyl palmitate (0·5%) + α-tocopherol (0·3%)		4	0·0152	0·00251	n.s.
β-Carotene (0·2%) + ascorbyl palmitate (0·3%)		1	0·0096	—	—

* P = probability value obtained by testing the significance of the difference between mean values of k for a particular solvent and for standard series of pure tricaprylin. n.s. = not significant.
ulc. = experiment in which some ulceration occurred (mice showing ulceration are not included in the results).

of cases. The other substances tested either had no effect on the rate of elimination or accelerated it. Surprisingly, both lecithin and kephalin were found to increase the rate of elimination in these experiments, in contrast to the earlier result (Weil-Malherbe and Dickens, 1946). On the whole, the reproducibility of results was found to be quite satisfactory, as shown by the k-values obtained for pure tricaprylin or for cholesterol, cholestanol or ascorbyl palmitate solutions in different experiments. The discrepancy in the case of the phospholipids must be attributed to real differences in the two preparations, both of which were rather crude. α-Tocopherol, in 0·3 per cent and still more in 1 per cent concentration, brought about a very pronounced acceleration of benzpyrene elimination which was little affected by the presence of lecithin, kephalin or cholesterol. Generally, no synergistic effects were observed with mixtures of antioxidants, but ascorbyl palmitate reduced to normal level the increase caused by lecithin or by lecithin + α-tocopherol.

Ethyl linoleate is included in Table V as an example of an autoxidizable lipid. At a concentration of 3 per cent in tricaprylin solution it had no effect on benzpyrene elimination. Pure ethyl linoleate as solvent for benzpyrene caused a slight, but probably significant acceleration. The mean k-value found in this series is, however, below the values calculated from the figures of Rusch, Mueller and Kline (1945), but the figures of these authors are generally higher than any found in this laboratory with lipoid solvents, ever since the factor of leakage and ulceration was adequately controlled. The difference may be partly accounted for by the practice of Rusch *et al.* of excising the tissues surrounding

TABLE VI.—*Elimination of Benzpyrene from Solutions Containing Sterols and Related Substances after Subcutaneous Injection into Mice.*

Addition in tricaprylin.	Concentration (%)	Total number of animals.	k (mean).	Standard error.	P.*
Cholesterol	3	6	0·0337	0·0068	<0·01
Cholestanol	3	5	0·0348	0·0026	<0·01
	10	5	0·0283	0·0020	<0·01
epi-Cholestanol	3 (ulc.)	3	0·0178	0·0085	n.s.
Coprostanol	3	5	0·0116	0·0020	n.s.
epi-Coprostanol	3	6	0·0155	0·0022	n.s.
Sitosterol (Roche)	3	5	0·0253	0·0057	0·02
"Phytosterol" (L. Light & Co.)	3	5	0·0112	0·0028	n.s.
Ergosterol	3	5	0·0136	0·0037	n.s.
7-Dehydrocholesterol	3	5	0·0100	0·0017	n.s.
Ergosterol peroxide	2	12	0·0174	0·0044	n.s.
Calciferol	0·2	6	0·0231	0·00737	n.s.
Deoxycholic acid	1 (suspension)	5	0·0221	0·0044	n.s.

* P = probability value obtained by testing the significance of the difference between mean values of k for a particular solvent and for standard series of pure tricaprylin. n.s. = not significant.

ulc. = experiment in which some ulceration occurred (mice showing ulceration are not included in results).

the injection area for analysis, a procedure which involves the risk of loss of injected material.

Table VI contains the results obtained with a number of sterols. In agreement with the previous result (Weil-Malherbe and Dickens, 1946) cholesterol was found to accelerate the elimination rate. The only sterol with a comparable effect was cholestanol. An acceleration of probable significance was also observed with sitosterol (Roche). None of the other compounds tested altered the rate of elimination significantly.

III. *Experiments on the Correlation of Elimination Rate and Carcinogenicity of* 3:4-*Benzpyrene.*

It was one of the objects of the survey described in the preceding section to find substances which would affect the elimination rate of benzpyrene in a positive or negative sense, and to test whether the change could be correlated with an inhibition or stimulation of tumour induction. In view of the discrepancy of the results obtained with the two different preparations of phospholipids their use seemed undesirable, as it indicated an insufficient control of all variables. There remained the choice between tocopherol and cholestanol as accelerators of benzpyrene elimination. The effect of tocopherol on tumour induction has already been studied by Rusch, Mueller and Kline (1945). The use of cholestanol therefore recommended itself. It was employed as a 3 per cent solution in tricaprylin.

The only substance with a probable inhibitory action on benzpyrene elimination was ascorbyl palmitate in 2 per cent solution. This was used for a second series of injections, although it was liable to cause ulceration.

A third series of mice was injected with benzpyrene in pure tricaprylin as a standard of comparison and as a check on the reproducibility of earlier results.

Glaxo FF mice of both sexes were used for the experiment. Groups of 55 mice were injected subcutaneously with the three different solvents, each animal receiving 0·3 ml. benzpyrene in 0·3 ml. solvent. One mouse of each series was sacrificed weekly for the analysis of its benzpyrene content.

Ulceration occurred in more than half of the mice injected with the solution of ascorbyl palmitate. None of the mice thus affected has been included in the final analysis, although two of them subsequently developed local tumours. Benzpyrene estimations on ulcer mice showed that the bulk of the injected benzpyrene was usually lost when ulceration occurred.

TABLE VII.—*Tumour Incidence after* 45 *Weeks.*

Solvent.	Effectual total of mice.	Number of local tumours.	% of local tumours.	Number of mice alive and tumour-free.	P.*
3% Cholestanol in tricaprylin	31	23	74	3	<0·01
Tricaprylin	36	14	39	11	
2% Ascorbyl palmitate in tricaprylin	17	7	41	5	..

* P = probability of difference being due to chance obtained by applying χ^2-test.

Tumour incidence.—The relevant data are contained in Table VII and Fig. 1. Whereas the curves for the tricaprylin and ascorbyl palmitate series are almost identical, the tumour incidence is increased in the cholestanol series. The difference between the cholestanol and tricaprylin series was evaluated by

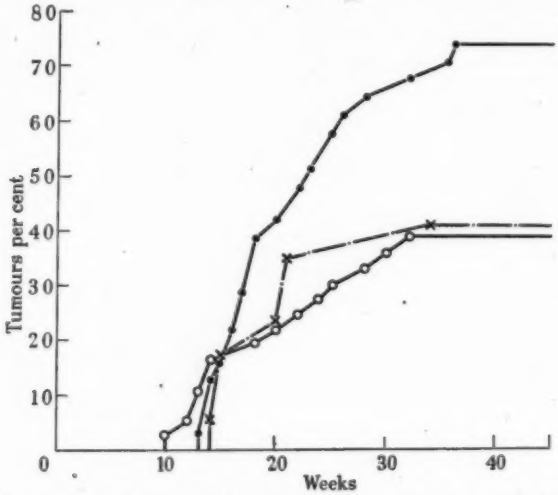

FIG. 1.—Incidence of local tumours following subcutaneous injection of 0·3 mg. 3:4-benzpyrene in the following solvents: (A) 3 per cent cholestanol in tricaprylin (solid circles); (B) tricaprylin (rings); (C) 2 per cent ascorbyl palmitate in tricaprylin (crosses).

the χ^2-test and was found to be statistically significant. The percentage incidence found in the tricaprylin series is in good agreement with the figures obtained in previous experiments (Weil-Malherbe and Dickens, 1944, 1946; Dickens and Weil-Malherbe, 1946). The latent period was about the same in all three series.

TABLE VIII.—*Elimination Constants of Benzpyrene in Different Solvents.*

Solvent.	Number of observations.	Linear regression coefficient.	Standard error.	P.*
3% Cholestanol in tricaprylin	14	−0·0371	0·00469	<0·05>0·02
Tricaprylin	16	−0·0222	0·00482	
2% Ascorbyl palmitate in tricaprylin	8	−0·00564	0·00162	<0·01

* P = probability value obtained by testing the significance of the difference between two regression coefficients.

Elimination of 3:4-benzpyrene.—The logarithms of S = the amount of benzpyrene remaining after t days have been plotted in Fig. 2 for the three series

of solvents used. The straight lines represent the linear regression curves calculated by the method of least squares. Their numerical values are given in Table VIII together with their standard error. Owing to the large proportion of ulceration in the ascorbyl palmitate experiment the number of animals was insufficient to continue the analyses for more than 50 days. Later, some analyses were carried out on mice which had developed tumours. The values of benzpyrene found at this time were, however, far below those which might have been expected if the elimination curve had continued linearly. It must

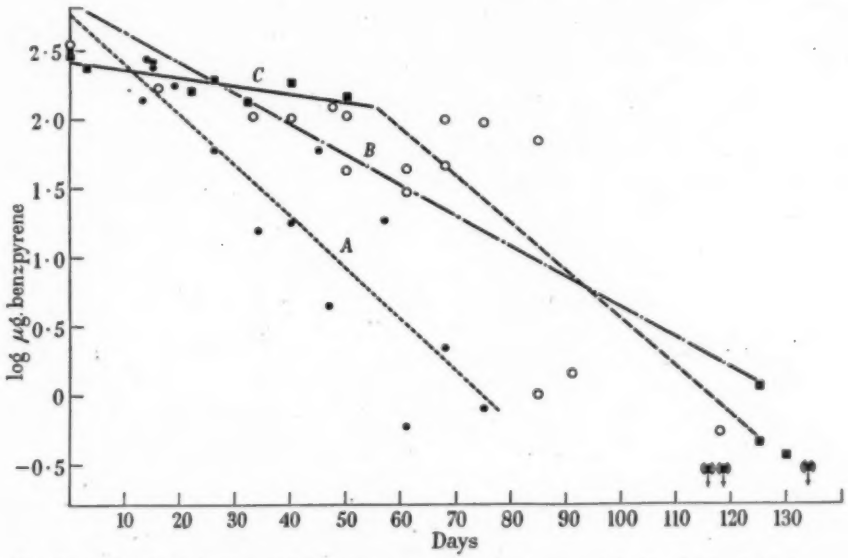

FIG. 2.—Rate of elimination of 3:4-benzpyrene after subcutaneous injection in the following solvents: (A) 3 per cent cholestanol in tricaprylin (dotted line; solid circles); (B) tricaprylin (line of dots and dashes; rings); (C) 2 per cent ascorbyl palmitate in tricaprylin (full line; solid squares. The broken line indicates the assumed elimination rate in the second phase. Bracketed squares represent analyses in which no measurable amounts of benzpyrene were detected).

be assumed that a change in the elimination rate had taken place in the interval. These late values were therefore not included in the calculation of the regression coefficient. The same tendency towards an accelerated elimination in the final phases of the elimination process can be discerned in the two other series, especially in the tricaprylin experiment. The inclusion of these late values is probably responsible for the fact that the constant was found to be somewhat higher than in previous experiments, though the difference is not significant.

Statistical analysis showed that the difference between the regression coefficients for the ascorbyl palmitate and the tricaprylin experiments was significant, that between the constants of the tricaprylin and the cholestanol experiment probably significant.

DISCUSSION.

In confirmation of previous results (Weil-Malherbe and Dickens, 1946) it has been found that the higher rate of benzpyrene elimination in the cholestanol series was also associated with a higher incidence of induced tumours. This correlation is further strengthened by the results of Rusch, Mueller and Kline (1945) on the effect of tocopherol. These authors found a stimulating effect on tumour induction by small amounts of tocopherol (0·1–0·2 mg.) in some experiments, though not regularly. As reported in this paper, 3 mg. tocopherol has a very pronounced accelerating effect on the rate of benzpyrene elimination. With 0·9 mg. the effect is still significant, though less intense. It would be interesting to investigate whether the procarcinogenic effect of tocopherol would be more regular if higher doses than those employed by Rusch *et al.* were used.

On the other hand, ascorbyl palmitate did not influence the incidence of tumours, although it inhibited the elimination of benzpyrene for a limited period. The results of benzpyrene analyses in this series seem to show that after 7–8 weeks the inhibition is followed by an activation of elimination. The latter phenomenon may cancel out any effects caused by the former, and the negative result obtained in this experiment is therefore not necessarily inconsistent with a correlation between low rate of elimination and low incidence of tumours.

The initial protection of benzpyrene by ascorbyl palmitate may be explained by the assumption of a higher affinity of the latter substance for the oxidant resulting in its preferential oxidation. The product of oxidation which is possibly a peroxide may itself catalyze the subsequent oxidation of benzpyrene. A similar process, though it is presumably much faster, may underlie the induced oxidation of benzpyrene in 80 per cent acetone containing autoxidizing ascorbic acid (Warren, 1943).

The effects of a number of sterols on the elimination of benzpyrene allow some tentative conclusions as to the mechanism of the activation caused by some of them.

A possibility which had to be considered was a pro-oxidant mechanism, similar to that assumed for the oxidation product of ascorbyl palmitate. Though a cholesterol peroxide is not known, other sterols, such as ergosterol or 7-dehydrocholesterol, are known to form peroxides, and a metabolic conversion of cholesterol to 7-dehydrocholesterol is quite feasible. However, not only the parent substances, ergosterol and 7-dehydrocholesterol, but also ergosterol peroxide itself were quite inactive.

If the structure of active and inactive sterols is considered as in the following comparison—

Active sterols.	*Inactive sterols.*
Cholestanol	*Epi*-cholestanol
Cholesterol	Coprostanol
(Sitosterol)	*Epi*-coprostanol
	" Phytosterol " (probably mainly stigmasterol)
	7-Dehydrocholesterol
	Ergosterol

it appears that the active substances have a trans I:II-, cis C_3:C_{10}-configuration and not more than one double bond. Substances with trans C_3:C_{10}- (*epi*-cholestanol) or with cis I:II-configuration (coprostanol) or with both (*epi*-coprostanol) as well as substances with more than one double bond (stigmasterol, 7-dehydrocholesterol and ergosterol) are inactive. This is exactly the situation found by Davis, Krahl and Clowes (1940) in their investigation on the interaction of benzpyrene and sterols in surface films. Their results indicate the formation of association complexes between benzpyrene and cholesterol or cholestanol at a water-air interface, whereas interaction is weak or absent with *epi*-cholestanol, coprostanol and ergosterol. The authors assumed that the hydrocarbon molecule is held between two appropriately oriented sterol molecules. The special steric configuration of cholestanol and cholesterol was thought to be more favourable to optimum packing of the molecules than the steric configuration of the inactive sterols.

This analogy is probably more than a mere coincidence. The association between sterol and hydrocarbon might decrease the hydrophobic properties and increase the surface activity of the hydrocarbon and thus account for its higher reactivity. The accelerated rate of elimination of benzpyrene probably indicates a more rapid metabolism of this substance, but it is still open to question whether this process is actually involved in the etiology of the cancerous change. The formation of association complexes between benzpyrene and sterols may at the same time lead to an easier accessibility of the oxidant and to a freer deployment of the forces or reactions responsible for carcinogenesis. Whether the two processes are linked or independent cannot be decided at present. A clear-cut correlation of delayed elimination with anticarcinogenesis would be more valuable evidence in this respect.

Deoxycholic acid, in spite of its eminent capacity for the formation of co-ordination compounds, increases the rate of elimination only slightly. This may be due to the low solubility of deoxycholic acid in tricaprylin. From an aqueous solution of benzpyrene and sodium deoxycholate the hydrocarbon is eliminated at a greatly increased rate (Weil-Malherbe, 1947).

SUMMARY.

(1) In experiments on the *in vitro* oxidation of 3:4-benzpyrene it is shown that addition of other substances may result in either a pro- or anti-oxidant effect according to the conditions. Thus phospholipids greatly increase the rate of oxidation of 3:4-benzpyrene by hydrogen peroxide in 80 per cent acetic acid, whereas lecithin alone or, to a greater extent, in combination with α-tocopherol inhibits the oxidation of benzpyrene by perbenzoic acid in tricaprylin solution. Addition of cholesterol has a similar, though smaller, effect.

(2) Mice were injected subcutaneously with tricaprylin solutions of 3:4-benzpyrene containing one or more of a number of additional compounds and the effect of these additions on the rate of benzpyrene elimination was studied. The substances tested mainly belonged to the two classes of antioxidants and sterols. The earlier observation of a delaying effect of phospholipids could not be confirmed with a new sample ; on the contrary, the new sample of phospholipids accelerated the elimination of benzpyrene. Otherwise the reproducibility of results, as far as it was tested, was satisfactory.

Ascorbyl palmitate in 2 per cent but not in 0·5 per cent solution was the only substance with a probable inhibitory effect, whereas α-tocopherol caused a marked acceleration. Amongst sterols, cholesterol, cholestanol and sitosterol increased the rate of elimination. Attention is drawn to the capacity of these sterols of forming association complexes with 3:4-benzpyrene in surface films and to the inactivity in this respect of those sterols which do not affect the rate of elimination of benzpyrene.

(3) The higher rate of benzpyrene elimination brought about by cholestanol (3 per cent solution in tricaprylin) is associated with a higher incidence of local sarcomas. The delay of benzpyrene elimination in the presence of ascorbyl palmitate (2 per cent solution in tricaprylin) is only temporary and lasts for about 6–7 weeks. It is probably followed by a phase of accelerated elimination. The addition of ascorbyl palmitate did not affect the incidence of induced tumours.

Grateful acknowledgment is made for the supply of samples of coprostanol, *epi*-coprostanol and *epi*-cholestanol by Dr. O. Rosenheim, F.R.S., and Dr. H. King, F.R.S., and for histological examinations by Dr. R. Schade.

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THE ELIMINATION AND CARCINOGENIC POTENCY OF 3:4-BENZPYRENE IN MICE AFTER SUBCUTANEOUS INJECTION OF NON-LIPOID SOLUTIONS.

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THE introduction of 1:3:7:9-tetramethyluric acid as a solubilizer for 3:4-benzpyrene (Weil-Malherbe, 1946) has made it possible to prepare aqueous solutions of benzpyrene suitable for injection. If solubilizer solutions of equal concentration (weight/volume) are compared, a tetramethyluric acid solution will dissolve about five times more benzpyrene than a caffeine solution and about 10 times more than a deoxycholate solution.

It has been suggested that the solubilization of hydrocarbons by purines is connected with the formation of coordination complexes (Weil-Malherbe, 1946). A similar mechanism was assumed to underlie the activation of elimination and carcinogenicity observed when benzpyrene is injected in solvents containing cholesterol or cholestanol (Weil-Malherbe, 1947). It was therefore of interest to study the rate of elimination and the carcinogenic activity of solubilized benzpyrene. It was anticipated that in the absence of a stable lipoid depot the dispersion of benzpyrene and its absorption by the surrounding tissues would be greatly speeded up. The additional effect of the solubilizer can only be gauged if allowance is made for the different physical conditions resulting from the absence of a lipoid depot. The rate of elimination after the injection of colloidal aqueous or ethereal solutions of benzpyrene was therefore also measured. Peacock and Beck (1938) obtained only a few tumours with 0·5–1 mg. benzpyrene subcutaneously administered to mice either as the powdered solid or in ethyl ether solution, and they attributed this to a more rapid dispersal and elimination of the hydrocarbon. The effects of aqueous solutions of benzpyrene made up with the aid of a solubilizer do not appear to have been adequately studied. Gummel and Rarei (1939) used sodium cholestenone sulphonate solutions as a solvent for benzpyrene, but their method of preparation (solution of benzpyrene in acetone, addition to cholestenone sulphonate solution and evaporation of acetone) suggests that benzpyrene may have been present partly in colloidal solution. They found after five months, as a result of daily injections in rabbits, chronic degenerative changes in parenchymatous tissues, such as amyloidosis of spleen and liver, necrosis of liver cells and testicular atrophy.

EXPERIMENTAL.

1:3:7:9-Tetramethyluric acid was prepared according to Biltz and Strufe (1916).

Tetramethyluric acid solutions of benzpyrene were prepared by shaking the

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purine solution with an excess of solid hydrocarbon for 3–4 hours at room temperature. If the concentration of tetramethyluric acid was higher than 3 per cent the mixtures were warmed on a water bath to about 50° C. and kept there for half an hour with constant stirring. Before use the solutions were filtered through Whatman No. 5 filter paper. Solutions containing 4 or 5 per cent tetramethyluric acid were kept at body temperature to prevent crystallization.

Deoxycholate solutions of benzpyrene were prepared in a similar way.

A colloidal solution of benzpyrene was prepared by the method of Feigenbaum (1944). It was filtered before use.

Benzpyrene was estimated fluorimetrically (Weil-Malherbe, 1944).

Glaxo FF mice of both sexes were used for the experiments.

Elimination Rates.

It was considered desirable to introduce as little variation of technique as possible. The same volume of solution as that used in the experiments with lipoid solvents, 0·3 ml., was therefore adhered to for the injections. A 5 per cent solution of tetramethyluric acid which, at body temperature, is practically saturated will dissolve about 0·2 mg. of benzpyrene in 0·3 ml., while 0·3 ml. of a 4 per cent solution contain about 0·15 mg. of benzpyrene. The 5 per cent solution, if injected in amounts of 0·3 ml., is appreciably toxic, causing a mortality of about 30 per cent, whereas the mortality is only about 7 per cent with the 4 per cent solution. The latter was therefore chosen for the elimination experiments.

A solution of sodium deoxycholate of the same concentration (4 per cent) only dissolves about 15 μg. benzpyrene per 0·3 ml. and, moreover, causes skin damage (haemorrhagic oedema followed by necrosis) in a number of cases. The subcutaneous injection of 0·3 ml. ether solution also proved rather toxic, and led to deep anaesthesia of all and the subsequent death of 21 out of 35 mice.

In all four series cases of skin necrosis were observed which developed near the site of injection after 2–4 weeks. The necrotic patch of skin usually showed a brilliant bluish-white fluorescence in ultraviolet light. A scab usually developed which was finally sloughed off. The fluorescent substance was lost in the process, and analyses showed that there was practically no benzpyrene left in such mice. Because of the prevalence of this phenomenon, especially in the ether series, the analyses had to be confined to the first 4–5 weeks. In the tetramethyluric acid series the majority of estimations performed after the eighth day showed that the benzpyrene content had fallen below the level of sensitivity of the method. To avoid an undesirable bias the estimations which gave a measurable result after this date were omitted from the statistical analysis, but they are shown in Fig. 1. In Fig. 1–4 the values of $\log S$ (= μg. benzpyrene found) were plotted against the time t (days after injection). The straight line represents the linear regression of $\log S$ on t, calculated by the method of least squares. The regression coefficients and their standard errors are given in Table I.

It is obvious that the rate of elimination in the two experiments in which solubilizers were used is of quite a different order from that observed in the other two experiments. The differences within these two groups are not

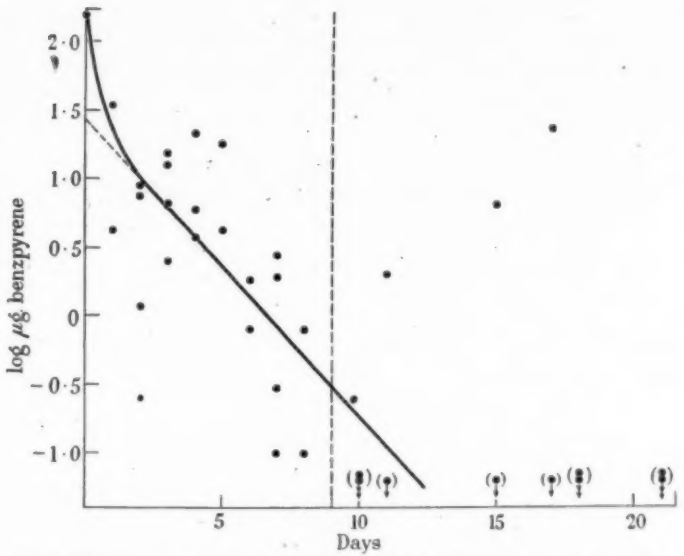

FIG. 1.—Elimination rate of 3:4-benzpyrene after subcutaneous injection of a saturated solution in 4 per cent aqueous tetramethyluric acid solution. Only the values to the left of the broken line were used for the calculation of the linear regression. Bracketed points represent analyses in which no measurable amounts of benzpyrene could be detected.

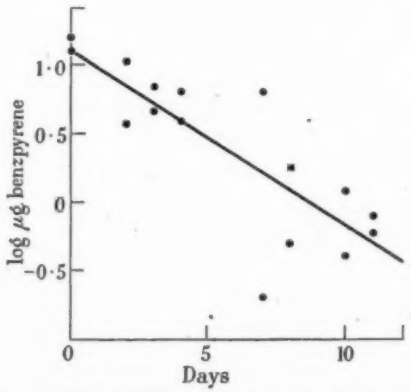

FIG. 2.—Elimination rate of 3:4-benzpyrene after subcutaneous injection of a saturated solution in 4 per cent aqueous sodium deoxycholate solution.

statistically significant ($P > 0{\cdot}05$). With ethereal or colloidal solutions the elimination proceeds at practically the same rate as was observed with various lipoid solvents, at least within the first 4–5 weeks. After this time ulceration may intervene and cause a sudden and almost complete loss of benzpyrene.

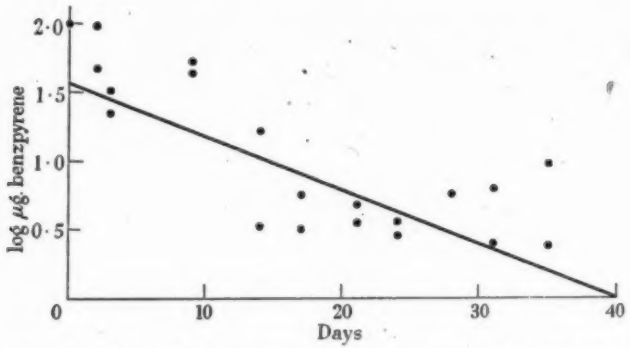

FIG. 3.—Elimination rate of 3:4-benzpyrene after subcutaneous injection of a colloidal solution.

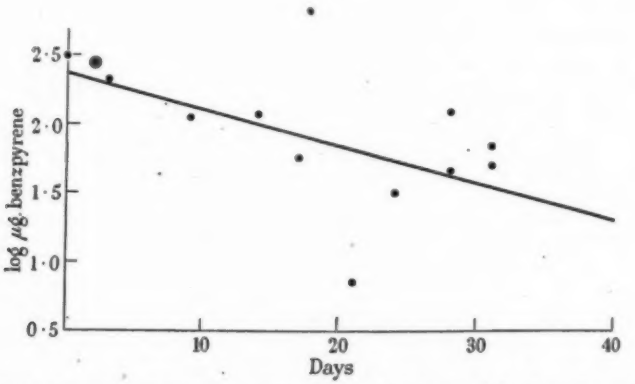

FIG. 4.—Elimination rate of 3:4-benzpyrene after subcutaneous injection of a solution in ethyl ether.

TABLE I.—*Elimination of 3 : 4-Benzpyrene Dissolved in Non-lipoid Vehicles After Subcutaneous Injection.*

Solution.	Initial amount of benzpyrene. (μg. in 0·3 ml.)	Number of observations.	Linear regression coefficient.	Standard error.
4% tetramethyluric acid	160	25	— 0·214	0·0532
4% sodium deoxycholate	14·3	16	— 0·127	0·0234
Colloidal	100	20	— 0·0388	0·00677
Ethyl ether	307	13	— 0·0264	0·00854

This may account for the impression expressed by Peacock and Beck (1938) that absorption and elimination was faster after injection of ether solutions of benzpyrene than it was after injection of lipoid solutions. On the other hand, there is a remarkable increase of the elimination rate in the presence of solubilizers,

amounting to an acceleration of about 5–10 times. In Fig. 5 the elimination rates of benzpyrene found in a number of experiments with lipoid and non-lipoid solvents are compared. The elimination constant of — 0·214 for tetramethyluric acid solution corresponds to a half-lifetime of 1·4 days, whereas the constant for tricaprylin, — 0·0157, corresponds to a half-lifetime of 19·1 days.

There is a rather wide scatter which becomes more marked as the elimination rate increases. In the tetramethyluric-acid series an initial divergence from linearity has to be assumed, indicating a rapid drop in the first few hours after injection to a level from which the further elimination proceeds in a linear fashion. Fairly high values of benzpyrene persist in a few sporadic cases even after the

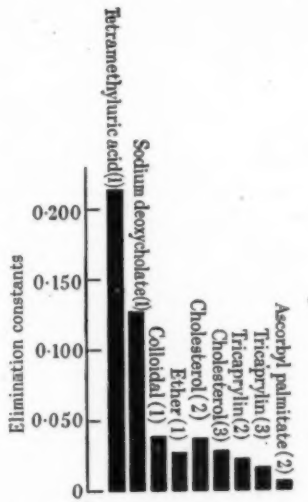

FIG. 5.—Elimination rates of 3:4-benzpyrene dissolved in different solvents after subcutaneous injection:
(1) From this paper.
(2) From Weil-Malherbe (1947).
(3) From Weil-Malherbe & Dickens (1946).

eighth day. These were cases showing necrotic skin areas impregnated with fluorescent material. Any benzpyrene trapped in the necrotic tissues presumably remains fixed there. If this interpretation is correct, the analyses of such cases are immaterial to the evaluation of the elimination constant and may be neglected.

Metabolism of Benzpyrene after Injection in Tetramethyluric Acid Solution.

In view of the increased elimination rate of benzpyrene after its injection in tetramethyluric acid solution the question arose whether a change in its metabolism took place. In particular the possibility of a urinary excretion of a complex between tetramethyluric acid and either unchanged or oxidized benzpyrene had to be considered. Twenty rats were therefore injected daily with

1 ml. of a 4 per cent solution of tetramethyluric acid saturated with benzpyrene for 12 days. An estimated total of 100 mg. benzpyrene was thus administered. The rats were kept in two metabolism cages, and their urine was collected in brown bottles containing 20 ml. 10 N sulphuric acid. The combined urine was filtered and extracted ten times with ether in a separating funnel. The pooled ether extracts were washed with water, dried and evaporated. After extraction the urine was neutralized with baryta, filtered and concentrated *in vacuo* to 300 ml. It was then extracted with chloroform in a separating funnel.

The residue from the ether extract was taken up in benzene, and the benzene solution extracted with a small volume of N sodium hydroxide solution. The soda extract was saturated with solid ammonium sulphate and re-extracted with benzene (phenolic fraction). Both benzene solutions, the phenolic and the neutral fraction, were dried and passed through columns of silica gel. The filtrates were then passed through columns of alumina and developed with benzene. No trace of unchanged benzpyrene or of benzpyrene quinones was detected in the neutral fraction. The phenolic fraction, after filtration through silica gel, showed a bluish-white fluorescence ; on the alumina column a band with yellow fluorescence formed and moved slowly down. This was taken as an indication of the presence of a phenolic metabolite, but the substance was present in traces only and could not be further characterized.

The benzene insoluble residue of the ether extract slowly crystallized. On further investigation, which need not be described in detail, it appeared mainly to consist of a mixture of methylmalonic (Boyland and Levi, 1936) and succinic acids.

Several crystalline fractions were also isolated from the chloroform extracts. Most of them were nitrogen-free and were not identified. Small quantities of two nitrogen-containing substances (m.p. 157 and 206° C.) were not identical with tetramethyluric acid.

The result of the metabolism experiment is in agreement with the prevailing views on the fate of injected benzpyrene, the bulk of which is apparently excreted with the faeces in the form of a phenolic derivative, and no unchanged benzpyrene and only traces of the phenolic metabolite pass into the urine (Berenblum and Schoental, 1943). The situation, at least as far as urinary excretion is concerned, does not seem to be modified by the presence of tetramethyluric acid. It is likely, therefore, that the increase of the elimination rate is largely a local effect, perhaps due to a more rapid penetration across the cell membranes. It is interesting to note that no unchanged tetramethyluric acid was found in the urine either. As is well known, methyluric acids may undergo partial or complete demethylation at one or several positions of the molecule within the body (Myers and Hanzal, 1946).

Carcinogenic Activity of 3:4-Benzpyrene Dissolved in Aqueous Tetramethyluric Acid Solution.

A series of mice were given a single injection of 0·3 ml. of a 5 per cent solution of tetramethyluric acid in water, saturated with benzpyrene. The amount of benzpyrene injected in this way was about 0·2 mg. per mouse. Among 21 mice alive after 8 months only one sarcoma arose locally, and this appeared 5 months after the injection.

In a second experiment a series of 20 mice were injected subcutaneously with 0·3 ml. of a 1 per cent aqueous solution of tetramethyluric acid saturated with benzpyrene, while an equal amount of a 1 per cent tetramethyluric acid solution free from benzpyrene was injected into another series of 20 mice. The injections were repeated twice weekly for 32 weeks. As a single dose contained about 10 μg. benzpyrene, each mouse received altogether about 0·32 mg. The experiment, which is now in its seventh month, has produced one local tumour so far.

DISCUSSION.

The results show two points of interest: (1) When benzpyrene is injected in colloidal or ethereal solution, its elimination proceeds at a rate not much different from that observed with several oily solutions (Fig. 5). The dispersal and absorption of benzpyrene do not therefore seem to be greatly facilitated by this form of application or, if they are, do not thereby appreciably accelerate the rate of elimination. Little is known about the carcinogenic response to these solutions. Morton and Mider (1939) observed in C 57 black mice an incidence of 50 per cent tumours after the subcutaneous injection of 0·25 mg. benzpyrene in colloidal solution, as compared with an incidence of 78 per cent after the injection of a sesame oil solution. It may be recalled that sesame oil has a procarcinogenic effect (Dickens and Weil-Malherbe, 1946); the incidence observed with the colloidal solution is close to the tricaprylin norm. Peacock and Beck (1938) found no tumours six months after the subcutaneous injection of mice with 0·5 mg. benzpyrene in 0·1 ml. ether. This is a surprising result. In the writer's experiments the injection of ethereal solutions led to frequent skin necrosis and demarcation, but no mention of it is made by Peacock and Beck. A reinvestigation with a careful watch for leakage or ulceration seems desirable.

(2) The elimination of benzpyrene is considerably faster when it is injected in an aqueous solution of a solubilizer than in a colloidal or ethereal solution. This result is unexpected for the following reasons: (1) The solubilization of benzpyrene increases with the square of the concentration of tetramethyluric acid (Weil-Malherbe, 1946) or sodium deoxycholate (Weil-Malherbe, unpublished) in the concentration range used. Precipitation is therefore caused by dilution, such as takes place when the injected solution mixes with the tissue fluids. (2) Addition of electrolytes also leads to precipitation. (3) The complexes between solubilizer molecules and benzpyrene molecules are assumed to be largely dissociated in solution (Weil-Malherbe, 1946), and a more rapid diffusion of the water-soluble solubilizer molecule in the tissues might be expected, a process which would also lead to precipitation of benzpyrene.

One would anticipate, therefore, that benzpyrene would be deposited and subsequently taken up in combination with, or solution by, tissue components in a similar manner, whether dissolved in ether, in colloidal solution or in an aqueous solution of a solubilizer. The difference in the rate of elimination, however, leads to the conclusion that this is not the case, and that the interaction between solubilizer and hydrocarbon persists to some extent after injection, presumably long enough in some way to assist the transport of the hydrocarbon across the cell membranes.

In the case of cholesterol and cholestanol a correlation has also been postulated between the increased rate of elimination of benzpyrene and the formation of association complexes (Weil-Malherbe, 1947). But, whereas here the speedier elimination was associated with a higher tumour incidence, the tumour incidence is negligible with tetramethyluric acid solutions of benzpyrene. A number of possible explanations offer themselves: (1) There may be a rate of metabolism of benzpyrene which is optimal for carcinogenesis, and this optimum may be exceeded in the case of tetramethyluric acid solutions. If this is so, not only the intensity, but also the duration of the process would be of importance. It need not be implied in this assumption that the metabolic reactions are a necessary cause of the carcinogenic stimulus; they may merely be a corollary to a simultaneous but unrelated activation of the reactions which initiate the carcinogenic metamorphosis. (2) The total quantities of benzpyrene injected or the effective concentrations in the tissue may have been insufficient. The dose used in the single-injection experiment was not a great deal below that which is found to be effective when applied in an oily vehicle. But a large proportion of it was eliminated during the first few hours after the injection. This sudden drop in benzpyrene content suggests that part of the injected fluid may have escaped into the circulation, and was therefore metabolized and eliminated at a much faster rate than the benzpyrene which remained in the subcutaneous tissues. In the mice which were given repeated injections of small doses of benzpyrene the chances of a sufficient local concentration being reached at the site of injection were of course smaller still. This was intentional, as it was the object of the experiment to induce not so much local as remote changes. However, the experiment will be repeated with higher doses.

Shear (1941) obtained only one local sarcoma out of 24 mice of Strains A and D, 16 months after injection of 0·5 ml. of a 0·25 per cent. aqueous solution of the sodium salt of the molecular compound formed by the combination of one molecule of 1:2:5:6-dibenzanthracene with four molecules of deoxycholic acid. A number of multiple lung tumours were observed in Strain A mice. The dose injected, 0·17–0·18 mg. dibenzanthracene, is effective if administered in an oily solvent. In similar experiments with an aqueous solution of the sodium salt of the 20-methylcholanthrene-deoxycholic acid compound, in which 0·5 ml. of a 0·25 per cent solution were injected (about 0·18 mg. methylcholanthrene per injection), two tumours arose amongst six Strain A mice and none in six Strain D mice after three months (Shear, 1936). At that time, and again after four months, the mice were reinjected with the same amount. After six months all Strain A mice and three Strain D mice had developed local tumours. This suggests that, provided the quantity is high enough in relation to the activity, the solubilization of carcinogenic hydrocarbons does not preclude their activity. (3) The solubilization of benzpyrene may bring about a qualitative change of its metabolism. Although the analysis of the urine of rats which had been injected with a benzpyrene-tetramethyluric acid solution gave no indication of a urinary excretion of unchanged benzpyrene or of substantial amounts of a metabolite, the rapid initial drop of benzpyrene content after injection is a digression from the usual course of events, whether the change concerns only the site or also the mechanism of the metabolic transformations.

It is at present impossible to decide which of these explanations is correct. But the three possibilities are by no means exclusive to each other and may all

apply together in varying degrees, with the second factor probably predominating.

SUMMARY.

Mice were subcutaneously injected with the following solutions of 3:4-benzpyrene : aqueous solutions made up with the aid of 1:3:7:9-tetramethyluric acid or sodium deoxycholate as solubilizers, an aqueous colloidal solution, and a solution in ethyl ether, and the rate of elimination of benzpyrene was measured. It was found that with the ethereal and colloidal solutions the rate of elimination was of the same order of magnitude as that observed with several lipoid solvents, while it was 5–10 times higher in the experiments in which solubilizers were used. The increase in the elimination rate brought about by tetramethyluric acid is not accompanied by a urinary excretion of unchanged benzpyrene or of substantial amounts of a metabolite, as shown by experiments on rats. No unchanged tetramethyluric acid was detected in the urine either.

Out of 21 mice injected with a single dose of a solution of tetramethyluric acid containing about 0·2 mg. benzpyrene per dose, only one developed a sarcoma after 5 months. One tumour arose in a series of 20 mice injected twice weekly for 32 weeks with a solution of tetramethyluric acid containing 10 μg. benzpyrene per injection.

The results have been discussed.

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SURVEY OF PAPERS.

KENNAWAY reviews the literature on the relation of cancer of the penis to circumcision. Cancer of the penis does not occur after circumcision on the 8th day according to Jewish practice, but occurs in later life in Moslems who are circumcised between the 3rd and 14th years. The failure of later circumcision to protect against cancer suggests that the train of events leading to malignant growth is set going early in life, and that removal of the cause does not then avert the development of cancer at a much later age (p. 335).

BUSK and CLEMMESEN find that among 4139 female cases of breast cancer notified to the Danish Cancer Registry there were 111 cases of cancer in the left breast to every 100 cases of cancer in the right breast. They refer to similar findings from Switzerland and England, and suggest that the difference in incidence of cancer may be related to a higher frequency of injury to the left breast (p. 345).

FAIRBURN and BURGEN describe a case of monocytic leukaemia in which a recurrent furunculosis preceded other leukaemic symptoms for several months. They discuss the pathological features and evolution of the various rashes encountered in monocytic leukaemia (p. 352).

FOULDS has found that mammary tumours arising in a strain of hybrid mice, presumably deficient in the milk factor, resemble the familiar mammary tumours in mice except that the growth of transplants of some of them is inhibited in male mice. His observations show that the growth of these transplanted tumours depends on a hormonal stimulus provided by the female and he suggests that this dependence characterises one phase in the progression of malignant mammary tumours in mice (p. 362).

SELBIE and ROBINSON have transmitted the Shope rabbit papilloma in series in the domestic rabbit. The lesions of the transmissible infection behave in a manner similar to those produced by the inoculation of extracts of cottontail papillomas in the domestic rabbit (p. 371).

BERENBLUM and SHUBIK confirm Mottram's finding that skin tumours in mice can be induced by a single application of 3:4-benzpyrene followed by repeated applications of croton oil, but have not confirmed his other observation that the application of croton oil previous to a single application of a carcinogen leads to an increase in the number of tumours induced (p. 379).

BERENBLUM and SHUBIK, using the method described in the preceding paper, find that when croton oil treatment is kept constant but different carcinogens are used the tumour incidence varies, but the average latent period remains constant. They also find that when the carcinogen is kept constant and croton oil treatment is delayed the tumour incidence remains the same, but there is a delay in the latent period corresponding to the length of the intervals free from treatment. They discuss the implications of their findings (p. 383).

HOCH-LIGETI finds that the carcinogenic activity of 2-acetaminofluorene is much reduced by hydroxylation, the appearance of tumours being delayed from about 200 days in rats receiving 2-acetaminofluorene to about 700 days in a group receiving 7OH-2-acetaminofluorene (p. 391).

ORR and BIELSCHOWSKY have studied the histology and histogenesis of pulmonary tumours in rats treated with 2-acetylaminofluorene and related compounds. The tumours are of two types, tubulo-papillary cuboidal-celled adenoma or carcinoma and squamous, and can both be traced back to an origin in the bronchiolar epithelium (p. 396).

COWEN finds that the number and size of lung adenomas produced in RIII, CBA and C57 mice by urethane decrease in that order, which corresponds to the incidence of spontaneous lung tumours (p. 401).

WEIGERT, CALCUTT and POWELL have investigated the metabolism of 3:4-benzpyrene in mouse skin up to 24 hours. The only metabolic product is BPX_2 which, after a lag of about 1 hour, increases rapidly and then declines slowly (p. 405).

WEIL-MALHERBE, in experiments on the oxidation of 3:4-benzpyrene, shows that the addition of other substances may result in either a pro- or anti-oxidant effect according to the conditions. He discusses his findings with reference to the relationship between the rate of elimination and the carcinogenic potency in mice of benzpyrene in various lipoid solvents (p. 410).

WEIL-MALHERBE has found that when mice are injected with ethereal and colloidal solutions of benzpyrene the rate of elimination of benzpyrene is of the same order of magnitude, whereas elimination is 5–10 times higher when solubilizers are used. However, the increased elimination produced by the addition of the solubilizer tetramethyluric acid is not associated with increased tumour incidence as with cholesterol and cholestanol (p. 423).